PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Charles E. Hart, Debra G. Gilbertson
Serial No.	:	10/664,432
Filed	:	September 19, 2003
For	:	METHODS FOR PROMOTING GROWTH OF BONE, LIGAMENT AND CARTILAGE
Confirmation No.	:	5728
Examiner	:	Jiang, Dong
Art Unit	:	1646
Docket No.	:	00-12D1
Date	:	October 23, 2007

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF STEPHEN R. JASPERS UNDER 37 C.F.R. § 1.132

Sir:

I, Stephen R. Jaspers, declare as follows:

1. I have been asked to serve as an expert in the field of genomics and molecular biology. My Curriculum Vitae, which recites my technical and educational expertise, is submitted hereto as Exhibit A.

2. I am presently a Principal Scientist, Autoimmunity and Inflammation, at ZymoGenetics, Inc.

3. I received a Ph.D. in Biochemistry, with a minor in Pharmacology, from the University of Arizona, Tucson, Arizona, in 1984. I served as a postdoctoral fellow and Research Assistant Professor in Biochemistry and Molecular Biology at the University of Massachusetts Medical Center, Worcester, Massachusetts, from 1984 to 1993. I have been in a variety of research positions at ZymoGenetics since 1993 to the present time, having been appointed as a Principal Scientist in 2001. As shown in my

Curriculum Vitae, I have been an author on many scientific publications and named as an inventor on several U.S. patents.

4. I have reviewed the claims and specification of U.S. Patent Application No. 10/664,432, entitled "Methods for Promoting Growth of Bone, Ligament and Cartilage" (hereinafter "the '432 application" or "the application").

5. I understand that the claims of '432 application are generally directed to methods for promoting growth bone, ligament, or cartilage using the growth factor domain portion of a protein now known as platelet-derived growth factor-C ("PDGF-C"). This protein has also been referred to in some scientific and patent documents as a member of the vascular endothelial growth factor (VEGF) family, and was variously called zvegf3; VEGF-F, and VEGF-E.

6. I further understand that independent claims 11 and 22 of the application read as follows:

> 11. A method for promoting growth of bone, ligament, or cartilage in a mammal comprising administering to said mammal a composition comprising:
>
> a pharmacologically effective amount of a dimeric protein comprising a first polypeptide chain disulfide bonded to a second polypeptide chain, each of said chains consisting of residues X-345 of SEQ ID NO:2, wherein X is an integer from 226 to 235, inclusive; and
>
> a pharmaceutically acceptable delivery vehicle.

> 22. A method for stimulating proliferation of osteoblasts or chondrocytes in a mammal comprising administering to the mammal a composition comprising:
>
> a pharmacologically effective amount of a dimeric protein comprising a first polypeptide chain disulfide bonded to a second polypeptide chain, each of said chains consisting of residues X-345 of SEQ ID NO:2, wherein X is an integer from 226 to 235, inclusive; and
>
> a pharmaceutically acceptable delivery vehicle.

7. I have also reviewed the Office Action dated August 22, 2007 ("Office Action"), issued by Examiner Jiang with respect to the '432 application.

8. I understand from the Office Action that the pending claims of the application stand rejected as allegedly obvious in view of Ferrara *et al.* (U.S. Patent No. 6,455,283; "Ferrara").

9. I have read and understand the Ferrara reference cited in the Office Action.

10. The statements set forth hereinbelow are offered to address the Examiner's remarks in the Office Action and to show that Ferrara does not teach or suggest a method as recited in the present claims of the '432 application.

11. PDGF-C has a two-domain structure that, as of December 7, 1998, was unique among the previously known PDGF family members, PDGF-A and PDGF-B. In addition to the growth factor domain at the C-terminus (also referred to as the "core PDGF domain"), PDGF-C includes an N-terminal CUB domain (CUB is an abbreviation of C1r/s, Uegf, and bone morphogenic protein-1 [BMP-1]), composed of about 110 amino acids from approximately residues 50 to 160 of the PDGF-C amino acid sequence. The CUB domain is followed by a hinge region of approximately 80 amino acids in length, linking the CUB domain to the growth factor domain.

12. PDGF-C was also unique among PDGFs that were known in the art as of December, 1998, in that PDGF-C is secreted from cells in a mitogenically inactive form, comprising both the growth factor domain and the CUB domain.

13. The PDGF-C growth factor domain by itself, in the absence of the CUB domain, is active as a high affinity agonist for PDGF receptor α ("PDGFRα"), while the full-length PDGF-C protein is not. In standard mitogenesis assays, PDGF-C is active only upon cleavage of the CUB domain from the growth factor domain (core PDGF) domain, as demonstrated, for example, by studies described in Li *et al.* (*Nature Cell Biol.* 2:302-309, 2000 (*see, e.g.*, p. 303 [1st col., 2nd para.] to p. 305 [1st col., top] and Figure 3), attached hereto as Exhibit B. Partial deletion of the N-terminus is inadequate to

generate an active fragment of PDGF-C, as shown by studies described by Fredriksson *et al.* (*J. Biol. Chem.* 280:26856-26862, 2005), attached hereto as Exhibit C. As demonstrated by Fredricksson *et al.*, even truncated variants of PDGF-C that lack the CUB domain, but retain a significant portion of the hinge region, are inactive. (*See* Fredriksson *et al.* at, e.g., p. 26859, Figure 2.)

14. A polypeptide chain as recited in claims 11 and 22 of the '432 application (a polypeptide chain "consisting of residues X-345 of SEQ ID NO:2, wherein X is an integer from 226 to 235, inclusive") corresponds to bioactive fragment of PDGF-C having the growth factor domain, but lacking the CUB domain and a significant portion of the hinge region. (*See, e.g.*, Li *et al.* at pp. 303-305; Fredriksson *et al.* at p. 26859, Figure 2.)

15. Ferrara discloses a human PDGF-C polypeptide, which they call "vascular endothelial growth factor-E (VEGF-E)," having the amino acid sequence set forth as Ferrara's SEQ ID NO:2 ("Ferrara's SEQ ID NO:2"). The amino acid sequence of Ferrara's SEQ ID NO:2 is 100% identical to SEQ ID NO:2 of the '432 application.

16. I understand the Office Action states the following with respect to Ferrara's disclosure:

> ...Ferrara teaches VEGF-E variants including [variants wherein] one or more amino acid residues are added, deleted, or substituted at the N- or C-terminus or within the sequence as well as *active fragments* thereof.
>
> [Office Action at page 3 (emphasis original; citing Ferrara at col. 8, ll. 15-24.]

17. I further understand column 8, lines 15-24 of Ferrara to state as follows:

> "VEGF-E variant" means an active VEGF-E polypeptide as defined below having at least 80% amino acid identity with the VEGF-E polypeptide having the deduced amino acid sequence shown in FIG. 2 for a full-length native-sequence VEGF-E polypeptide. Such VEGF-E polypeptide variants include, for instance, VEGF-E polypeptides wherein

> one or more amino acid residues are added, deleted, or substituted at the N- or C-terminus of the sequence of FIG. 2 or within the sequence as well as active fragments thereof.
>
> [Ferrara at col. 8, ll. 15-24.]

18. The above cited disclosure of Ferrara does not teach or suggest any particular active fragments of PDGF-C, nor does this disclosure otherwise provide any specific guidance as to which fragments of PDGF-C would be active.

19. Even when the entire Ferrara disclosure is considered, Ferrara does not teach or suggest, whether explicitly or implicitly, a fragment of PDGF-C as recited in claim 11 or 22 of the '432 application. Ferrara does not teach or suggest a polypeptide fragment comprising the core PDGF-C growth factor domain in the absence of the PDGF-C CUB domain, including a polypeptide chain "consisting of residues X-345 of SEQ ID NO:2 [of the '432 application], wherein X is an integer from 226 to 235, inclusive." Moreover, Ferrara does not specifically teach or suggest a unique, two-domain structure for PDGF-C containing an active growth factor domain. In particular, Ferrara does not teach the approximate boundaries of the growth factor domain of Ferrara's SEQ ID NO:2, nor does Ferrara disclose or suggest that proteolytic cleavage from the inactive precursor of an N-terminal region, comprising the CUB domain and a significant portion of the hinge region, releases the active growth factor domain from the full-length protein.

20. I understand that the Office Action states the following with regard to the person of ordinary skill in the art:

> [I]t would have been obvious to the person of ordinary skill in the art at the time the invention was made to make the composition composition comprising the homodimer of the polypeptide fragments as defined in the instant claims … based on the sequence of VEGF-E taught by Ferrara.
>
> [Office Action at page 3, bottom.]

21. Contrary to the above-referenced statement, in view of the state of the art of the PDGF family of growth factors known in the art as of December, 1998, a skilled artisan reading Ferrara would not have been led to a fragment of PDGF-C as recited in claim 11 or 22 of the '432 application. Previous to December, 1998, there was no disclosure or suggestion of a PDGF having a two-domain structure as observed for PDGF-C and which is secreted in mitogenically inactive form, as previously summarized above (*see* ¶¶ 11-13). Therefore, as of December, 1998, in view of Ferrara's lack of any teaching or suggestion regarding the two-domain structure of PDGF-C, the significance of this structure with respect to activation, and the absence in the art of other PDGFs having these characteristics, Ferrara would not have specifically suggested to a person of ordinary skill in the art to modify Ferrara's polypeptide of SEQ ID NO:2 to achieve a fragment of PDGF-C as recited in claim 11 or 22 of the '432 application.

22. For at least the reason that Ferrara does not teach or suggest a fragment of PDGF-C as recited in claims 11 and 22 of the '432 application, Ferrara also does not teach or suggest a method for using such a PDGF-C fragment to promote the growth of bone, ligament, or cartilage, or to stimulate proliferation of osteoblasts or chondrocytes, as recited in these claims.

23. I further declare that statements made herein of my knowledge are true, and that all statements made on information are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: Oct 23, 2007

By:
Stephen R. Jaspers

Stephen R. Jaspers

ADDRESS

Home: 5829 150th PL SW
Edmonds, WA 98026

Office: ZymoGenetics, Inc.
1201 Eastlake Ave East
Seattle, WA 98102

E-mail: sjas@zgi.com

Phone: 425-743-2406

Phone: 206-442-6735
FAX: 206-442-6608

PROFESSIONAL EXPERIENCE

ZymoGenetics, Inc. (1993-present)

Principal Scientist, Autoimmunity and Inflammation, 2001-present
Principal Scientist, In Vitro Biology, 1998-2001
Director, Biology Network, 1996-1998
Assoc. Director, Diabetes Research, 1995-1996
Senior Scientist, Diabetes Research, 1994-1995
Scientist, Diabetes Research, 1993-1994

University of Massachusetts Medical Center, Worcester, MA (1984-1993)

Research Assistant Professor, Department of Biochemistry and Molecular Biology, 1991-1993
Regulation of Glycogen Metabolism and the Function of Phosphoprotein Phosphatases
in Hormone Signal Transduction

Assistant Director, Peptide Synthesis/Antibody Production Core Facility 1992-1993,
Coordination of peptide-protein conjugate synthesis for use in antibody production

Postdoctoral Research Associate, Department of Biochemistry and Molecular Biology 1984-1991
Sponsor: Dr. Thomas B. Miller, Jr. Regulation of glycogen metabolism in heart and liver

East Acres Biologicals, Southbridge, MA (1985-1993)

Technical Advisor/ Protein Chemist. 1985-1993
Coordination and production of peptide-protein conjugate synthesis for use in antibody production

Department of Social and Health Services, State of Washington, Regional Pesticide Control Laboratory, Wenatchee, WA (1978-1979)

Laboratory Assistant-Technician, 1978-1979(summers) Director: Dr. A. Robbins.

EDUCATION

Ph.D, Biochemistry (Minor: Pharmacology) (1984)
University of Arizona, Tucson, AZ
Advisor: Dr. Marc E. Tischler. Metabolic Responses of Skeletal Muscle to Hypokinesia/Hypodynamia

B.S. Biochemistry(1980)
Washington State Unversity, Pullman, WA
Advisor: Dr. Michael Griswold. The effect of hormones on rat Sertoli cells in vitro. Testosterone metabolism in transformed cells.

OTHER TRAINING

University of Washington Executive Program Courses, Seattle
Negotiation Skills
Strategic Management of Technology and Innovation

HONORS AND AWARDS

1997	Eureka Award(Outstanding Scientific Discovery), ZymoGenetics, Inc.
1987	Postdoctoral National Research Fellowship Award, National Institute of Diabetes and Digestive and Kidney Diseases
1985	Department of Biochemistry Dissertation Award, University of Arizona

ISSUED US PATENTS:

Hexokinase II promoter and assay methods. US 5,741,704 April 21, 1998
Testis-specific insulin homolog proteins US 5,959,075 September 28, 1999
Human Prohormone Convertase 4 US 6,013,503 January 11,2000
Polynucleotides encoding insulin homolog zins3 US 6,046,028 April 4, 2000
Human Prohormone Convertase 4 US 6,100,041 August 8, 2000
Methods for stimulating pancreatic islet cell regeneration US 6,114,307 September 5, 2000
Human Prohormone Convertase 4 US 6,127,162 October 3, 2000
Testis-specific insulin homolog polypeptides US 6,183,991 February 6, 2001
Beta-1,3-galactosyltransferase homolog, ZNSSP6 US 6,361,985 March 26, 2002
Nucleic acids encoding connective tissue factor homologs US 6,395,890 May 28, 2002
Short gastrointestinal peptides US 6,420,521 July 16, 2002
Beta-1,3,galactosyltransferase homologs US 6,416,988 July 9,2002
TML peptides US 6,627,729 September 30, 2003
Antibodies that bind testis-specific insulin homolog polypeptides US 6,709,659 March 23, 2004
Zins1 polypeptide composition stimulating pancreatic islet growth US 6,737,509 May 18, 2004
Motilin Homologs US 6,838,438 January 4, 2005
Zsig33-like peptides US 6,897,286 B2 May 24, 2005
Polynucleotides encoding motilin homologs US 6,939,690 B2 September 6, 2005
Antibodies that bind testis-specific insulin homolog polypeptides US 7,070,774 July 4, 2006

PUBLICATIONS

Kuestner RE, Taft DW, Haran A, Brandt CS, Brender T, Lum K, Harder B, Okada S, Ostrander CD, Kreindler JL, Aujla SJ, Reardon B, Moore M, Shea P, Schreckhise R, Bukowski TR, Presnell S, Guerra-Lewis P, Parrish-Novak J, Ellsworth JL, Jaspers S, Lewis KE, Appleby M, Kolls JK, Rixon M, West JW, Gao Z, Levine SD
Identification of the IL-17 Receptor Related Molecule IL-17-RC as the Receptor for IL17F. J Immunology 2007 179: 5462-5473.

Parrish-Novak J. Xu X., Brender T, Yao L., Jones C, West J, Brandt C, Jelinek L, Madden K, McKernan PA, Foster DC, Jaspers S, Chandrasekher YA. Interleukins 19, 20, and 24 signal through two distinct receptor complexes. Differences in receptor-ligand interaction mediate unique biological functions. J Biol Chem. 2002 Dec 6;277(49):47517-23.

Xu W, Presnell SR , Parrish-Novak J, Kindsvogel W, Jaspers S, Chen Z, Dillon SR, Gao Z , Gilbert T, Madden K, Schlutsmeyer S, Yao L, Whitmore TE, Chandrasekher Y, Grant FJ, Maurer, M, Jelinek L, Storey H, Brender T, Hammond A, Topouzis S, Clegg CH, Foster DC. A soluble class II cytokine receptor, IL-22RA2, is a naturally occurring IL-22 antagonist. Proc Natl Acad Sci 2001 98: 9511-9516

Lok S, Johnston DS, Conklin D, Lofton-Day CE, Adams R, Jelmberg A, Whitmore TE, Schrader S, Griswold MD, Jaspers SR. Identification of INSL6, a new member of the insulin family that is expressed in the testis of the human and rat. Biol. Reproduction. 2000 62: 1593-1599

Conklin D, Lofton CE, Haldeman BA, Ching A, Whitmore TE, Lok S, Jaspers SR. Identification of INSL5, a new member of the insulin superfamily. Genomics 1999 60: 50-56

Whitmore TE, Maurer MF, Day HL, Jelmberg AC, Dasovich MM, Sundborg LM, Burkhead SK, Heipel MD, Madden KL, Dramer JM, Kuijper JL, Xu WF, Jaspers SR, Holly RD, Lok S. The assignment of the human insulin receptor-related receptor gene (INSRR) to chromosome 1q21->q23 by the use of radiation hybrid mapping. Cytogenet Cell Genet 1999 87: 93-94

McIntosh, CHS, Bremsak I, Lynn FC, Gill R, Hinke SA, Gelling R, Nian C, McKnight G, Jaspers S, Pederson RA. Glucose-dependent insulinotropic polypeptide stimulation of lipolysis in differentiated 3T3-L1 cells: wortmannin-sensitive inhibition by insulin. Endocrinology 1999 140: 398-404

Baltensperger K, Kozma LM, Jaspers SR, Czech MP. Regulation by insulin of phosphatidylinositol 3'-kinase to alpha- and beta-isoforms of p85 regulatory subunit. J Biol Chem 1994 269(46): 28937-46

Miller C, Rulfs J, Jaspers SR, Buckholt M, Miller, Jr TB. Transformation of adult ventricular myocytes with temperature sensitive A58(tsA58) mutant of the SV40 large T antigen. Mol. Cell. Biochem. 1994 136(1): 29-34

Wadzinski BE, Wheat WH, Jaspers S, Peruski Jr. LF, Lickteig RL, Johnson GL Klemm DJ Nuclear protein phosphatase 2A dephosphorylates protein kinase A-phosphorylated CREB and regulates CREB transcriptional stimulation. Mol Cell Biol 1993 13(5): 2822-34

Jaspers SR, Garnache AK, Miller, Jr. TB Factors affecting the activation of glycogen synthase in primary culture cardiomyocytes. J Mol Cell Cardiol 1993 25(10): 1171-8

Buczek-Thomas JA, Jaspers S, Miller, Jr TB Post-receptor defects account for the phosphorylase hypersensitivity in cultured diabetic cardiomyocytes. Mol Cell Biochem 1992 117: 63-70

Buczek-Thomas JA, Jaspers S, Miller, Jr. TB Adrenergic activation of glycogen phosphorylase in primary culture diabetic cardiomyocytes. Am. J. Physiol. 1992 262: H649-H653

Pallas DC, Weller W, Jaspers S, Miller, Jr. TB, Lane WS, Roberts TM. The third subunit of protein phosphatase 2A, a 55 Kda protein which is apparently substituted for by T antigens in complexes with 36 and 63 Kda PP2A subunits, bears remarkably little resemblance to T antigens. J. Virol. 1992 66: 886-893

Matic G, Jaspers S, Miller TB, and Michelson A. Platelet glycogen synthase: Further characterization and effect of insulin. Thrombosis Res. 1991 63: 427-432

Corvera S, Jaspers S, Pasceri M. Acute inhibition of insulin-stimulated glucose transport by the phosphatase inhibitor, okadaic acid. J. Biol. Chem. 1991 266: 9271-9275

Pallas D, Druker B, Mamon H, Li P, Shahrik L, Martin B, Brautigan D, Jaspers S, Miller T, Serunian L, Cantley L, Schaffhausen B, Roberts T. Interactions of polyoma virus T antigens with host cell proteins. in: Advances in Applied Biotechnology Series, Vol. 7; Gene Regulation and AIDS, Transcriptional Activation, Retroviruses and Pathogens. 1991 pp.95-106. Portfolio Publishing Co., Woodlands, TX.

Miller Jr.TB, Jaspers S The effects of diabetes on glycogen metabolism in ventricular cardiomyocytes. in: The Diabetic Heart, M. Nagano and N.S. Dhalla(eds.), Raven Press, Ltd., New York, 1991 pp. 323-337

Karlund, JK, Jaspers SR, Khalaf N, Bradford AP, Miller TB, Czech MP. An insulin-stimulated kemptide kinase purified from rat liver is deactivated by phosphatase 2A. J. Biol. Chem. 1991 266: 4052-4055

Jaspers SR, Miller Jr. TB. Purification and the immunological characterization of rat protein phosphatase type 2A: enzyme levels in diabetic heart and liver. Mol. Cell. Biochem 1991 101: 167-174.

Hsu SD, Jaspers SR, Davis BB, Cardell Jr. RR, Miller, Jr. TB, Drake RL. Appearance of a nonfunctional form of hepatic glycogen synthase in late gestation. Arch. Biochem. Biophys 1990
281(1): 152-156

Pallas DC, Shahrik LK, Martin BL, Jaspers S, Miller TB, Brautigan DL, Roberts TM Polyoma middle and small T antigens and SV40 small T antigen form stable complexes with protein phosphatase 2A. Cell 1990 60: 167-176

Tischler ME, Henriksen EJ, Jaspers SR, Jacob S, Kirby C. Changes in muscles accompanying non-weight-bearing and weightlessness. Advances in myochemistry 1989 2, 325-338

Rulfs J, Jaspers SR, Garnache AK, Miller, Jr. TB. Phosphorylase synthesis in diabetic hepatocytes and cardiomyocytes. Am. J. Physiol. 1989 257(20): E74-E80

Jaspers SR, Henriksen EJ, Gater D, Tischler ME. Effects of immobilization on amino acids in non-weight-bearing rat soleus muscles. Metabolism 1989 38: 303-310

Jaspers SR, Rulfs J, Johnson GL, Mole J, Miller, Jr. TB. Amino-terminal sequence analysis of rat heart and muscle glycogen synthase: Homology to the rabbit enzyme and the implications for hormonal control. Archives of Biochemistry and Biophysics 1989 268(2): 630-636

Jaspers SR, Jacob S, Henriksen E, Tischler ME. Metabolism of branched chain amino acids in unloaded leg muscles from intact and adrenalectomized rats. Metabolism 1989 38(2): 109-114

Jaspers S, Tischler ME Insulin effects on amino acid uptake by unloaded rat hindlimb muscles. Hormone and Metabolic Research 1988 20: 125

Jaspers SR, Fagan JM, Satarug S, Cook PH, Tischler ME Effects of immobilization on rat hindlimb muscles under non-weight bearing conditions. Muscle & Nerve 1988 11: 458-466

Johnson GL, Brautigan DL, Shriner C, Jaspers S, Arino J, Mole JE, Miller, Jr. TB, Mumby M. Sequence homologies between type 1 and 2A protein phosphatases. Molecular Endocrinology 1987 1: 745-748

Wolleben CD, Jaspers SR, Miller, Jr. TB. Use of adult rat cardiomyocytes to study cardiac glycogen metabolism. Am. J. Physiol. 1987 252: E673-E678

Jaspers SR, Tischler ME Role of glucocorticoids in the response of rat leg muscles to reduced activity. Muscle & Nerve 1986 9: 554-561

Jaspers SR, Tischler ME Metabolism of amino acids by atrophied soleus of tail-casted suspended rats. Metabolism 1986 35(3): 216-223

Tischler ME, Henriksen EJ, Jacob S, Cook, P, Jaspers SR Response of rat hindlimb muscles to 12 hours recovery from tail-cast suspension. The Physiologist 1985 28(suppl.): S129-S130

Tischler ME, Jaspers SR, Henriksen, EJ, Jacob S. Responses of skeletal muscle to unloading- a review. The Physiologist 1985 28(suppl.): S13-S15

Jaspers SR, Fagan JM, Tischler ME. Biochemical response to chronic shortening in unloaded soleus muscles. J. Appl. Physiol: Respirat. Environ. Exercise Physiol. 1985 59(4): 1159-1163

Jaspers SR, Tischler ME. Atrophy and growth failure of rat hindlimb muscles in tail-cast suspension. J. Appl. Physiol: Respirat. Environ. Exercise Physiol. 1984 57(5) 1472-1479

Tischler ME, Jaspers SR, Fagan JM Prevention of metabolic alterations caused by suspension hypokinesia in rats. The Physiologist 1983 26(suppl.): S98-S99

Tischler ME, Jaspers SR Synthesis of amino acids in weight bearing and non-weight bearing leg muscles in suspended rats. The Physiologist 1982 25(suppl.): S155-S156.

PDGF-C is a new protease-activated ligand for the PDGF α-receptor

Xuri Li*, Annica Pontén*, Karin Aase*, Linda Karlsson†, Alexandra Abramsson†, Marko Uutela‡, Gudrun Bäckström§, Mats Hellström†, Hans Boström†, Hong Li*, Philippe Soriano¶, Christer Betsholtz†, Carl-Henrik Heldin§, Kari Alitalo‡, Arne Östman§ and Ulf Eriksson*#

*Ludwig Institute for Cancer Research, Stockholm Branch, Box 240, S-17177 Stockholm, Sweden
†Department of Medical Biochemistry, University of Göteborg, Medicinaregatan 9A, S-41390 Göteborg, Sweden
‡Molecular/Cancer Biology Laboratory, Haartman Institute, University of Helsinki, PO Box 21 (Haartmaninkatu 3), SF-00014 Helsinki, Finland
§Ludwig Institute for Cancer Research, Uppsala Branch, Box 595, S-75124 Uppsala, Sweden
¶Program in Developmental Biology, Division of Basic Sciences, A2-025, Fred Hutchinson Cancer Research Center, PO Box 19024, Seattle, Washington 98109-1024, USA
#e-mail: ueri@licr.ki.se

Platelet-derived growth factors (PDGFs) are important in many types of mesenchymal cell. Here we identify a new PDGF, PDGF-C, which binds to and activates the PDGF α-receptor. PDGF-C is activated by proteolysis and induces proliferation of fibroblasts when overexpressed in transgenic mice. *In situ* hybridization analysis in the murine embryonic kidney shows preferential expression of PDGF-C messenger RNA in the metanephric mesenchyme during epithelial conversion. Analysis of kidneys lacking the PDGF α-receptor shows selective loss of mesenchymal cells adjacent to sites of expression of PDGF-C mRNA; this is not found in kidneys from animals lacking PDGF-A or both PDGF-A and PDGF-B, indicating that PDGF-C may have a unique function.

Platelet-derived growth factors are important in connective tissue growth, survival and function, and consist of disulphide-linked dimers involving two polypeptide chains, PDGF-A and PDGF-B. PDGFs are members of the PDGF/vascular endothelial growth factor (PDGF/VEGF) family of growth factors, which at present consists of seven different members. For almost two decades, PDGF homodimers (PDGF-AA and PDGF-BB) and the heterodimer (PDGF-AB) were thought to be the only ligands for the PDGF α-receptor (PDGFR-α) and the PDGF β-receptor (PDGFR-β), two receptor tyrosine kinases that are expressed by many cultured cell lines grown *in vitro* and by mesenchymal cells *in vivo*[1]. PDGF-B binds to both PDGFRs, whereas PDGF-A selectively binds to PDGFR-α. PDGFs regulate cell proliferation, survival and chemotaxis *in vitro*[2]. *In vivo*, they function in a paracrine mode as they are often expressed in epithelial (PDGF-A) or endothelial (PDGF-B) cells in close apposition to the PDGFR-expressing mesenchyme[3]. In tumour cells and in cell lines grown *in vitro*, co-expression of PDGFs and their receptors may also generate autocrine loops resulting in cellular transformation[4,5]. Moreover, overexpression of PDGF has been observed in several pathological conditions, including malignancies, atherosclerosis and fibroproliferative diseases[6].

The importance of PDGFs as regulators of cell proliferation and survival is evident from gene-targeting studies in mice that show distinct physiological functions of different PDGFs and their receptors, despite the overlapping ligand specificities of PDGFR-α and PDGFR-β[7,8]. Homozygous null mutations in genes encoding either of the two PDGF polypeptides or the receptors are lethal. About 50% of mice lacking PDGF-A exhibit a lethal phenotype before day 10 of embryonic development (E10), while surviving animals exhibit a complex postnatal phenotype including a lack of alveolar-septum formation in the lungs[9], dermal and hair-follicle defects[10], reduced proliferation of oligodendrocyte progenitors and hypomyelination of the central nervous system[11]. The phenotype of mice lacking PDGFR-α is more severe, with incomplete cephalic closure, impaired neural-crest development, cardiovascular and skeletal defects and oedemas, leading to embryonic death around E8–16 (ref. 12). Mice lacking PDGF-B and those deficient in PDGFR-β develop similar phenotypes that are characterized by renal, haematological and cardiovascular abnormalities and death at E17–19 (refs 13–15). The renal and cardiovascular defects are due, at least in part, to a lack of proper recruitment of mural cells (vascular smooth-muscle cells, pericytes or mesangial cells) to blood vessels[13,15,16].

Deletion of the PDGFR-α gene in mice results in certain defects, such as cleft face and spina bifida, that are not seen in homozygous deletions of the genes for PDGF-A, PDGF-B, or both (M.H., C.B. and P.S., unpublished observations; see below). This implies the existence of further PDGFR-α ligands. Here we identify and characterize PDGF-C, a new PDGFR-α ligand. PDGF-C is a member of the PDGF/VEGF family of growth factors with a unique domain organization and expression pattern. The identification of a new PDGFR-α ligand indicates that control of PDGFR-α signalling may be more complex than was previously supposed.

Results

Primary structure of human PDGF-C. In a BLAST search of the expressed-sequence tag (EST) databases at the National Center for Biotechnology Information, we identified a mouse EST (accession number AI020581) and a partially overlapping human EST (W21436), which encoded a protein with significant similarities to several members of the PDGF/VEGF family of growth factors. We isolated complementary DNA clones from a human fetal lung cDNA library and their nucleotide sequences were determined. The full-length cDNA encoded a polypeptide of 345 amino acids (Fig. 1a), the hydrophobic amino terminus of which has features indicative of a signal sequence. A putative site for signal-peptidase cleavage is located between amino acids 22 and 23; cleavage here results in a secreted protein with a length of 323 amino acids and a relative molecular mass (M_r) of 36,774 (Fig. 1b). Three putative *N*-linked glycosylation sites, located at positions 25, 55 and 254, are found in the full-length protein (Fig. 1a, marked in green). As this new member of the PDGF/VEGF family binds to one of the PDGFRs (see below), we have named it PDGF-C.

PDGF-C has a unique two-domain structure (Fig. 1b). After the signal sequence is a separate N-terminal domain composed of about 110 amino acids (residues 50–160) with homology to CUB domains. These domains were first found in complement subcomponents C1r/C1s, urchin EGF-like protein and bone morphogenetic protein-1 (BMP-1; ref. 17). Homology searches showed that

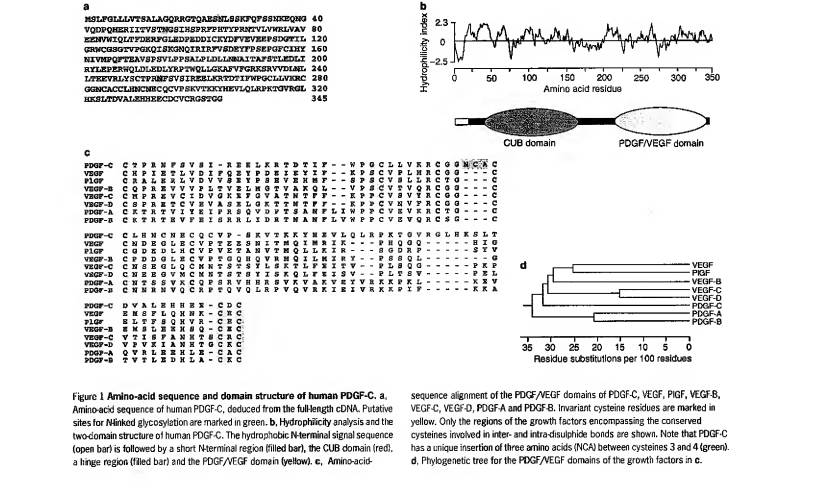

Figure 1 **Amino-acid sequence and domain structure of human PDGF-C. a**, Amino-acid sequence of human PDGF-C, deduced from the full-length cDNA. Putative sites for *N*-linked glycosylation are marked in green. **b**, Hydrophilicity analysis and the two-domain structure of human PDGF-C. The hydrophobic N-terminal signal sequence (open bar) is followed by a short N-terminal region (filled bar), the CUB domain (red), a hinge region (filled bar) and the PDGF/VEGF domain (yellow). **c**, Amino-acid-sequence alignment of the PDGF/VEGF domains of PDGF-C, VEGF, PlGF, VEGF-B, VEGF-C, VEGF-D, PDGF-A and PDGF-B. Invariant cysteine residues are marked in yellow. Only the regions of the growth factors encompassing the conserved cysteines involved in inter- and intra-disulphide bonds are shown. Note that PDGF-C has a unique insertion of three amino acids (NCA) between cysteines 3 and 4 (green). **d**, Phylogenetic tree for the PDGF/VEGF domains of the growth factors in **c**.

the CUB domain in PDGF-C shares 27–37% identity with the prototypic CUB domains in C1r/C1s and BMP-1 (data not shown).

In PDGF-C, the CUB domain is followed by a hinge region 80–90 amino acids in length (residues 161–250) and finally by the C-terminal PDGF/VEGF domain. This latter domain shares 27–35% identity with corresponding regions of PDGFs and VEGFs. A characteristic of the PDGF/VEGF domain is a pattern of eight invariant cysteine residues involved in interchain and intrachain disulphide bonding. All of these cysteines are found in PDGF-C, but their spacing is different to that in previously identified PDGF/VEGF domains. Alignment of the amino-acid sequences of PDGF/VEGF domains in PDGF-C, PDGF-A, PDGF-B and several VEGFs showed that an insertion of three extra residues (sequence NCA) has occurred in PDGF-C between cysteines 3 and 4 (Fig. 1c; insertion is marked in green). In addition to the eight invariant cysteine residues found in all members of the PDGF/VEGF family, four extra cysteines are found in the PDGF/VEGF domain of PDGF-C. These non-conserved cysteine residues are located between invariant cysteines 3 and 4, 5 and 6, 6 and 7, and beyond the eighth conserved cysteine. Phylogenetic analysis of PDGF/VEGF domains showed that PDGF-C is more similar to VEGFs than to PDGFs (Fig. 1d).

PDGF-C is a PDGFR-α agonist. We transfected COS-1 cells with cDNA encoding full-length PDGF-C. We collected secreted PDGF-C in serum-free medium and subjected it to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions, and immunoblotting using an antiserum against a peptide from the PDGF-C sequence. We also analysed conditioned medium from mock-transfected COS-1 cells as a control. Full-length PDGF-C migrated as a species of estimated M_r 55,000 (55K) under reducing conditions; detectable levels of PDGF-C were not secreted by mock-transfected cells (Fig. 2a). The principal SDS–PAGE migrant was larger than the size of PDGF-C estimated from the amino-acid sequence, indicating that PDGF-C may be glycosylated but not proteolytically processed before secretion.

To investigate the biological properties of PDGF-C, we produced, in baculovirus-infected insect cells, the full-length protein and a version of PDGF-C containing only the PDGF/VEGF domain (residues 230–345, hereafter referred to as the core domain). We purified histidine-tagged versions of these PDGF-C constructs and subjected them to SDS–PAGE under both reducing and non-reducing conditions. Both proteins were generated as disulphide-linked homodimers (Fig. 2b). Full-length PDGF-C migrated as species of M_r 90K and 55K under non-reducing and reducing conditions, respectively, whereas the core domain of PDGF-C migrated as species of M_r 32K and 23K, respectively. Thus it seems that PDGF-C, like PDGF-A and PDGF-B, forms a disulphide-bonded dimer, PDGF-CC.

We investigated the receptor specificity of PDGF-CC by using full-length and core-domain versions of PDGF-CC as competitors in PDGFR ligand-binding assays. We determined the ability of PDGF-CC, at increasing concentrations, to compete with the binding of ^{125}I-labelled PDGF-BB to cells expressing PDGFR-α or PDGFR-β (Fig. 3a, b). The core domain of PDGF-CC, but not the full-length protein, efficiently competed with PDGF-BB for binding to PDGFR-α, but not for binding to PDGFR-β. In similar experiments involving binding of ^{125}I-labelled PDGF-AA to cells expressing PDGFR-α, the core domain of PDGF-CC also competed with PDGF-AA for binding to PDGFR-α (data not shown).

To demonstrate its direct binding to PDGFR-α, we radiola-

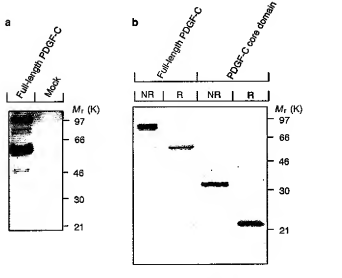

Figure 2 **Expression and purification of full-length and core-domain PDGF-C.** **a**, Full-length PDGF-C is expressed as a principal species of M_r55K in transfected COS-1 cells. COS-1 cells were transfected with an expression vector for PDGF-C or with an empty vector (Mock). TCA-precipitated proteins in serum-free conditioned medium were subjected to SDS–PAGE under reducing conditions and immunoblotted using an anti-peptide antiserum. **b**, Expression and purification of full-length and core-domain PDGF-C in baculovirus-infected Sf9 cells. The His_6-tagged proteins were purified on Ni–NTA–agarose; aliquots were subjected to SDS–PAGE under non-reducing (NR) and reducing (R) conditions. Proteins were stained with Coomassie brilliant blue. Both full-length PDGF-C and the PDGF-C core domain are present as disulphide-linked homodimers of M_r90K and 32K, respectively. Under reducing conditions, full-length and core-domain PDGF-C migrated as species of M_r55K and 23K, respectively.

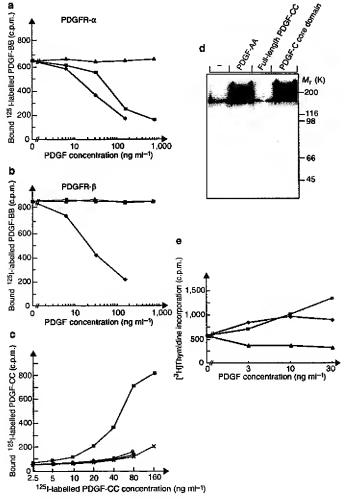

Figure 3 **The core domain of PDGF-CC, but not full-length PDGF-CC, is a high-affinity agonist for PDGFR-α.** Competitive inhibition of binding of ^{125}I-labelled PDGF-BB to porcine aortic endothelial (PAE) cells expressing PDGFR-α (**a**) or PDGFR-β (**b**) by increasing concentrations of PDGF-BB (diamonds), full-length PDGF-CC (triangles) or the core domain of PDGF-CC (squares). **c**, Binding of ^{125}I-labelled PDGF-CC core domain to PAE cells expressing PDGFR-α (squares and crosses) and PDGFR-β (circles and diamonds), in the presence (crosses and diamonds) or absence (squares and circles) of a 100-fold molar excess of unlabelled PDGF-CC core domain, respectively. **d**, Induction of tyrosine phosphorylation of PDGFR-α by full-length and core-domain PDGF-CC (100 ng ml^{-1}) in PAE cells expressing PDGFR-α. Unstimulated PAE cells (–), and cells stimulated with PDGF-AA (10 ng ml^{-1}) were used as controls. PDGFR-α was immunoprecipitated from detergent-lysed cells, subjected to SDS–PAGE and immunoblotted with monoclonal PY20 antibodies against phosphotyrosine. **e**, Induction of cell proliferation of human foreskin fibroblasts treated with full-length PDGF-CC (triangles) or the core domain of PDGF-CC (squares), as measured by incorporation of [^{3}H]thymidine into cellular DNA. Cells treated with PDGF-AA (diamonds) were used as a positive control.

belled the core domain of PDGF-CC using the Bolton–Hunter reagent. Radiolabelled PDGF-CC, at increasing concentrations, bound to cells expressing PDGFR-α in a saturable way, with a half-maximal concentration of 35–50 ng ml^{-1}; no specific binding was observed with cells expressing PDGFR-β (Fig. 3c). The binding of the radiolabelled PDGF-CC core domain was readily competed by an unlabelled version of the core domain but not by full-length PDGF-CC. A k_d of 1.1±0.2 nM was calculated for binding of the PDGF-CC core domain to PDGFR-α, which is similar to the k_d values for binding of both PDGF-AA and PDGF-BB to PDGFR-α[16]. Cross-competition experiments showed that binding of ^{125}I-labelled PDGF-CC core domain was efficiently competed by unlabelled PDGF-AA and PDGF-BB (data not shown).

We investigated the ability of PDGF-CC to induce tyrosine phosphorylation of a receptor. We stimulated cells expressing PDGFR-α with 100 ng ml^{-1} PDGF-CC, and subjected the immunoprecipitated PDGFR-α to SDS–PAGE and immunoblotting using the monoclonal anti-phosphotyrosine antibody PY20. We also analysed unstimulated and PDGF-AA-stimulated cells as controls. The core domain of PDGF-CC efficiently induced tyrosine phosphorylation of PDGFR-α, as did PDGF-AA (Fig. 3d). PDGFR-α from unstimulated cells or from cells stimulated with full-length PDGF-CC showed only background levels of activation. Subsequent probing of the filters with antibodies against PDGFR-α confirmed that equal amounts of PDGFR-α were present in all lanes (data not shown).

To verify that PDGF-CC was able to induce cellular DNA synthesis mediated by PDGFR-α, we treated serum-starved human foreskin fibroblasts expressing PDGFR-α with PDGF-CC and measured stimulation of cell proliferation as the level of incorporation of [^{3}H]thymidine into cellular DNA. We used cells stimulated with PDGF-AA as a positive control. PDGF-AA and the core domain of PDGF-CC both stimulated cellular DNA synthesis, whereas full-length PDGF-CC was unable to do so (Fig. 3e). The results from these four sets of experiments show that the core domain of PDGF-CC, but not full-length PDGF-CC, is a specific PDGFR-α agonist similar in potency to classical PDGFs. Other results (our unpublished observations) show that the CUB domain, expressed as a single domain in baculovirus-infected insect cells, does not compete for binding of either PDGF-AA or the core

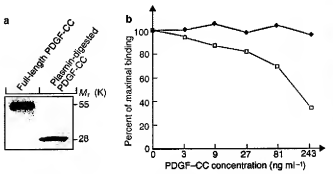

Figure 4 **Limited proteolysis of full-length PDGF-CC releases an active C-terminal PDGF/VEGF domain. a**, SDS–PAGE and immunoblotting analysis of full-length and plasmin-digested PDGF-CC under reducing conditions. Full-length PDGF-CC and the protected fragment of M_r28K were observed by immunoblotting using an anti-peptide antiserum against an internal peptide located in the N-terminal part of the PDGF/VEGF domain of PDGF-C. **b**, Inhibition of binding of ^{125}I-labelled PDGF-CC core domain to porcine aortic endothelial cells expressing PDGFR-α by increasing amounts of plasmin-digested (open squares) or full-length (closed diamonds) PDGF-CC.

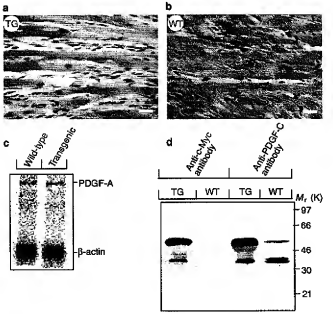

Figure 5 **Transgenic expression of PDGF-C in mouse heart. a**, Transgenic (TG) expression of PDGF-C in the mouse heart, using the promoter for the α-myosin heavy chain, induces strong proliferation of interstitial cells such as cardiac fibroblasts. Misorganization of myofibres is probably secondary to the extensive growth of the interstitium. **b**, Control section from a wild-type (WT) mouse heart showing the normal appearance of the myocardium. Tissue sections in **a** and **b** were stained with haematoxylin/eosin. Scale bars represent 20μm. **c**, RNase-protection analysis of transcripts encoding PDGF-A in wild-type and PDGF-C-transgenic hearts. Protected fragments corresponding to PDGF-A (320 bp) and to β-actin, the internal control (250 bp), are shown. **d**, SDS–PAGE and immunoblotting analysis of transgenic and wild-type PDGF-C in mouse heart. Transgenic PDGF-C was tagged at the C terminus with a c-Myc epitope and observed using a specific monoclonal antibody against this tag. Total PDGF-C was detected using an anti-peptide serum against mouse PDGF-C.

domain of PDGF-CC to PDGFR-α. Similarly, recombinant CUB domain does not induce tyrosine phosphorylation of PDGFR-α, nor does it affect the ability of PDGF-AA or the core domain of PDGF-CC to activate PDGFR-α.

We have also analysed the ability of the core domain of PDGF-CC to bind to the VEGF receptors. These analyses showed no significant interactions of core domain PDGF-CC with VEGF receptors 1, 2 or 3 (our unpublished observations).

Activation of full-length PDGF-CC by limited proteolysis *in vitro*. The ability of the core domain of PDGF-CC, but not of full-length PDGF-CC, to bind to and activate PDGFR-α raises the possibility that the N-terminal CUB domain may sterically prevent the PDGF/VEGF domain from interacting with the receptor, and that proteolytic removal of the CUB domain may be a necessary step to allow the biological activation of PDGF-CC. When we subjected full-length PDGF-CC to limited proteolysis using various proteases such as plasmin, thrombin, urokinase-type plasminogen activator, chymotrypsin and trypsin, a fragment of M_r28K was generated by plasmin, but none of the other tested proteases, as revealed by SDS–PAGE under reducing conditions and immunoblotting using internal anti-peptide antiserum (Fig. 4a). Affinity-purified antibodies against the core domain of PDGF-CC recognized a fragment of the same size, whereas similar analysis using an antiserum towards N-terminal peptides failed to reveal any protected fragment (data not shown). We therefore concluded that the M_r28K fragment contained the reduced form of the core domain of PDGF-CC.

To investigate whether protease treatment could activate PDGF-CC, we used increasing concentrations of untreated or plasmin-treated full-length PDGF-CC to compete with radiolabelled PDGF-CC core domain in PDGFR-α binding assays. Plasmin-digested PDGF-CC, but not the untreated (full-length) version, efficiently competed for binding to PDGFR-α (Fig. 4b). Control experiments confirmed that relevant concentrations of plasmin, present in the plasmin-treated fractions of full-length PDGF-CC, did not affect the ability of cells expressing PDGFR-α to bind to ligands (data not shown). These results show that proteolytic cleavage of the full-length protein, removing the N-terminal CUB domain, can generate a receptor-binding ligand from full-length PDGF-CC.

PDGF-C induces connective-tissue proliferation in mouse heart. To investigate whether full-length PDGF-C is proteolytically processed and is able to induce a proliferative response *in vivo*, we over-expressed PDGF-C tagged with c-Myc epitope in the hearts of transgenic mice, using the α-myosin heavy chain (α-*MHC*) promoter[19]. The hearts of the transgenic animals exhibited a progressive cardiac hypertrophy, and analysis of tissue sections showed that enforced expression of PDGF-C induced a strong proliferation of myocardial interstitial cells such as cardiac fibroblasts (Fig. 5a, b). RNase protection assays showed that levels of PDGF-A transcripts were not upregulated in the transgenic hearts (Fig. 5c). Cardiac fibroblasts express PDGFR-α, and PDGF-AA is a potent mitogen for this cell type[20], indicating that the observed expansion of the interstitium may result from the overexpression of PDGF-C. Expansion of the interstitium in the transgenic hearts caused a drastic misorganization of cardiac myofibres, implying that the functional properties of the transgenic hearts were severely compromised (a detailed characterization of the transgenic animals will be presented elsewhere).

The hyperproliferation of cardiac fibroblasts caused by overexpression of PDGF-C implies that proteases capable of converting full-length PDGF-CC into an active species are expressed in the myocardium. To test this hypothesis, we analysed tissue extracts from normal and transgenic hearts by immunoblotting using an antibody against the c-Myc epitope to detect transgenic PDGF-C, and a rabbit anti-peptide antiserum to the core domain of mouse PDGF-C to detect both endogenous and transgenic PDGF-C (Fig. 5d). Analysis under reducing conditions showed abundant expression of full-length (M_r52K) c-Myc-tagged PDGF-C in transgenic hearts and the presence of several processed species, including a prominent fragment of M_r34K, and analysis using the anti-peptide antiserum to the PDGF-CC core domain confirmed these results.

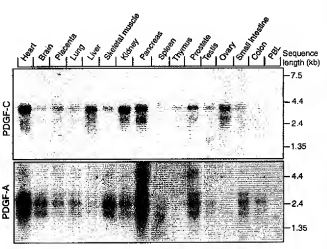

Figure 6 **Expression of transcripts encoding PDGF-C and PDGF-A in human tissues.** A northern blot using several tissues was sequentially hybridized with ^{32}P-labelled probes for PDGF-C (upper panel) or PDGF-A (lower panel). Abundant expression of PDGF-C transcripts was seen in heart, liver, kidney, pancreas and ovary. Prominent co-expression with PDGF-A transcripts was seen in heart and pancreas. PBL, peripheral blood leukocytes.

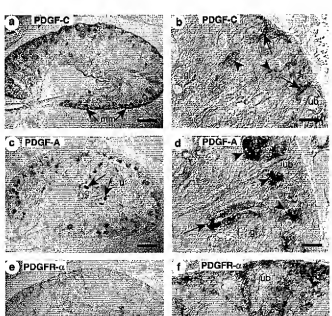

Figure 7 **Expression of PDGF-C, PDGF-A and PDGFR-α in the developing mouse kidney.** Non-radioactive *in situ* hybridization, showing the expression of transcripts for PDGF-C (**a**, **b**), PDGF-A (**c**, **d**) and PDGFR-α (**e**, **f**) in E16.5 mouse kidneys. Blue staining was observed against the unstained background using differential-interference-contrast optics. PDGF-C expression is seen in the metanephric mesenchyme (mm; **a**) and seems to be upregulated in the condensed mesenchyme undergoing epithelial conversion (**b**, arrows), which is situated on either side of the ureter bud (ub). PDGF-C expression remains at lower levels in early nephronal epithelial aggregates (**b**, arrowheads), but is absent from mature glomeruli (gl) and tubular structures. PDGF-A is expressed in early nephronal epithelial aggregates (**d**, arrowheads), but once the nephron has developed further, PDGF-A expression is restricted to the developing Henle's loop (**d**, arrow). Strongest expression is seen in the Henle's loops in the developing marrow (**c**, arrows). The branching ureter (u) and the ureter bud (ub) are negative for PDGF-A. PDGFR-α is expressed throughout the mesenchyme of the developing kidney (**e**, **f**). White hatched lines in **b**,**d**, **f** outline the cortex border. Scale bars represent 250μm in **a**, **c**, **e** and 50μm in **b**, **d**, **f**.

These results indicate that enzymes capable of proteolytically processing latent full-length PDGF-CC *in vivo* may be expressed in the myocardium, and that activated PDGF-CC may be able to promote proliferation of cardiac fibroblasts expressing PDGFR-α.

Expression of PDGF-C mRNAs in human tissues. Northern blotting showed that PDGF-C is encoded by a major transcript of length 3.8–3.9 kilobases (kb), and a minor one of 2.8 kb. Highest levels of expression were observed in heart, liver, kidney, pancreas and ovary cells (Fig. 6, upper panel). Smaller amounts of the mRNAs were observed in most other tissues, including placenta, skeletal muscle and prostate, but they were undetectable in spleen, colon and peripheral-blood leukocytes. In comparison, PDGF-A transcripts were abundant in heart and pancreas, whereas lower levels were observed in most other tissues including brain, placenta, lung, skeletal muscle, kidney and prostate; no signals were obtained from liver, thymus, or peripheral-blood leukocytes (Fig. 6, lower panel). The principal transcripts encoding PDGF-A are 1.45, 2.4 and 3.0 kb in length. As expected, prominent co-expression of the two PDGFs was observed in the heart and pancreas, whereas PDGF-C expression was predominant in the liver, kidney and ovary. PDGF-A expression predominated in brain, skeletal muscle, prostate, small intestine and colon tissues.

Localization of PDGF-C mRNA in the mouse embryo. We carried out an *in situ* hybridization using E14.5–17.5 mouse embryos. Several epithelial sites of PDGF-C expression were found, notably in ducts connected to the epidermis, such as the urethra and salivary-gland ducts, and in developing epidermal openings, such as the mouth, nostrils, ears and eyelids (data not shown; a detailed analysis of the expression pattern will be presented elsewhere). At these and other sites, PDGF-C expression occurred in close proximity to sites of PDGFR-α expression, and was distinct from the expression sites of both PDGF-A and PDGF-B. This implies that PDGF-C may activate PDGFR-α *in vivo*, and may have functions that are not shared with PDGF-A or PDGF-B.

One of the sites at which PDGF-C expression was strongest was the developing kidney (Fig. 7a, b). Here PDGF-C was expressed in the metanephric mesenchyme, most notably in aggregates undergoing epithelial conversion as a prelude to tubular development[11]. PDGF-A expression was not observed in these early aggregates, but was strong in later stages of tubular development (Fig. 7c, d), particularly in the developing Henle's loop. Thus the expression patterns of PDGF-C and of PDGF-A in the developing nephron are spatially and temporally distinct. PDGF-C is expressed in the earliest stages (mesenchymal aggregates) and PDGF-A in the latest stages (formation of Henle's loop) of nephron development. PDGFR-α was expressed throughout the mesenchyme of the developing kidney (Fig. 7e, f) and may therefore be targeted by both PDGF-C and PDGF-A. PDGF-B expression is also seen in the developing kidney, but it occurs only in vascular endothelial cells[15]. PDGFR-β expression takes place in the perivascular mesenchyme, and its activation by PDGF-B is crucial for recruitment of mesangial cells into glomeruli[12–15].

As the expression pattern of PDGF-C in the developing kidney indicated a possible function in kidney development, distinct from those of PDGF-A and PDGF-B, we compared the kidney histology at E16.5 of mice lacking PDGFR-α with that of wild-type mice, mice lacking PDGF-A and mice lacking both PDGF-A and PDGF-B. Mice deficient for PDGFR-α have a defective kidney phenotype, not seen in those lacking PDGF-A or both PDGF-A and PDGF-B,

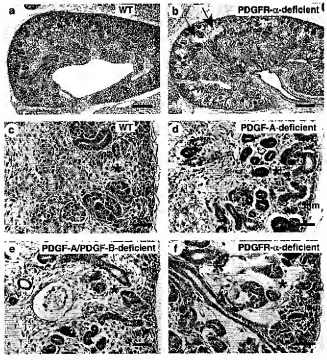

Figure 8 Loss of interstitial mesenchyme in kidneys lacking PDGFR-α, but not in those lacking PDGF-A or both PDGF-A and PDGF-B. Histology at E16.5 of wild-type kidneys (WT; **a**, **c**), and of kidneys lacking PDGFR-α (**b**, **f**), PDGF-A (**d**), or both PDGF-A and PDGF-B (**e**). Note the lack of interstitial mesenchyme in the cortex of kidney deficient in PDGFR-α (**b**, arrows; **f**, asterisk) and the presence of interstitial mesenchyme in all other genotypes (**c**, **d**, **e**, asterisk). The branching ureter (u) and the metanephric mesenchyme (mm) and its epithelial derivatives appear normal in all mutants. The abnormal glomerulus in kidneys lacking both PDGF-A and PDGF-B reflects the failure of mesangial-cell recruitment into the glomerular tuft resulting from the absence of PDGF-B[31,32]. Scale bars represent 250μm in **a**, **b** and 50μm in **c**–**f**.

consisting of a marked loss of interstitial mesenchyme in the developing kidney cortex (Fig. 8). The cells lost in this phenotype are therefore cells that express PDGFR-α adjacent to the sites of PDGF-C expression, potentially reflecting a loss of PDGF-C signalling.

Discussion

We have identified a new member of the PDGF/VEGF family of growth factors as a ligand for PDGFR-α and elucidated at least one of its functions. Given that classical PDGFs and their receptors have been extensively studied for more than a decade, the identification of a new PDGF ligand was highly unexpected.

PDGF-C has a two-domain structure not previously observed in this family of growth factors, with an N-terminal CUB domain and a C-terminal PDGF/VEGF-homology domain. The structure of the PDGF/VEGF domain in PDGF-C shares a low overall sequence identity with other PDGF/VEGF domains, although the eight invariant cysteine residues involved in the formation of intermolecular and intramolecular disulphide bonds are present. The spacing of cysteine residues in the central, most highly conserved region of this domain is different from that in other PDGF/VEGF domains, with an insertion of three amino-acid residues. Despite the fact that the insertion occurs close to the loop-2 region that is thought to be involved in receptor binding, our data show that this domain of PDGF-CC binds to PDGFR-α with an almost identical affinity to that of PDGF-AA or PDGF-BB. In addition, four extra cysteine residues are present in this domain. Whether these are involved in further disulphide bonding remains to be established. However, the altered spacing of the cysteines in the PDGF/VEGF-homology domain of PDGF-C may influence its capacity to form heterodimers with other PDGF chains, although we have so far failed to obtain any evidence of heterodimerization between PDGF-C and the classical PDGFs (our unpublished observations).

One reason that PDGF-C was not previously identified may be that it is synthesized and secreted as a latent growth factor, requiring proteolytic removal of the N-terminal CUB domain for receptor binding and activation. The active PDGF/VEGF domain is thought to be stabilized by several disulphide bonds forming a cysteine knot[22], indicating that removal of the N-terminal domain may be unlikely to induce a new receptor-binding epitope. Instead the CUB domains are thought to sterically block the receptor-binding epitopes in the unprocessed dimer. This idea is supported by two lines of evidence, from studies of the PDGF-CC core domain and of plasmin-treated full-length PDGF-CC. Furthermore, analysis of endogenous and transgenic PDGF-C in heart tissue showed that the protein is processed *in vivo*, and that ectopic expression induces a strong proliferative response in cardiac fibroblasts expressing PDGFR-α. Proteins proteolytically processed *in vitro* and *in vivo* are devoid of N-terminal fragments of M_r greater than 16K–18K, as determined by SDS–PAGE. These data are consistent with loss of the 110-amino-acid CUB domain and part of the hinge region between the CUB and core domains. Overall, our results indicate that PDGF-CC, devoid of the CUB domains, may act as a ligand for PDGFR-α. The extent to which the length of the hinge region may modulate the biological effects of PDGF-CC remains to be seen. A partially processed PDGF-CC dimer, with only one chain carrying the CUB domain, could perhaps act as an antagonistic intermediate that is still able to bind to a receptor, but unable to induce receptor dimerization and subsequent activation.

The protease(s) that activates PDGF-C *in vivo* remains to be identified. PDGF-CC is not proteolytically processed during secretion in transfected COS cells and most of the transgenic PDGF-CC was full-length. These data, and the fact that PDGF-C contains the accessory CUB domain found in several other extracellular proteins, indicate that proteolytic removal of the CUB domain may occur extracellularly and not during secretion. This is in contrast to the processing of PDGF-A and PDGF-B[33], which seems to be carried out intracellularly by furin-like endoproteases[34].

The function of the N-terminal CUB domain in PDGF-C is unclear apart from its ability to act as an inhibitory domain for receptor binding. This structural domain is present in several membrane-bound and secreted proteins, often as multiple copies[35] (cubulin/intrinsic-factor receptor, for example, contains 27 copies[36]). CUB domains can be involved in protein–protein and protein–carbohydrate interactions and in binding of low-M_r ligands[37–39]. Accessory domains in the PDGF/VEGF family are often important in extracellular deposition of these factors; examples are the basic C-terminal motifs present in PDGF-B and in certain splice isoforms of PDGF-A, VEGF, VEGF-B and placental growth factor (PlGF), which are thought to mediate binding to the extracellular matrix[21–25]. The silk domains of VEGF-C and VEGF-D probably also have a similar function[26]. The CUB domain of PDGF-C may bind to the pericellular matrix, as well as having an inhibitory function in receptor binding and activation. It may also influence signalling mediated by PDGFR-α, by binding to putative co-receptors.

The defective heart phenotype induced in transgenic animals expressing PDGF-C, involving an expansion of the cardiac interstitium, is reminiscent of cardiac hypertrophy and fibrosis. Cardiac fibroblasts synthesize the extracellular matrix and have a key function in adaptation and remodelling of the interstitium, typically seen following myocardial infarctions and increased haemodynamic load. Given that PGDF-C is normally expressed in heart, our observations indicate that PDGF-C may be important in physiological and pathophysiological remodelling of the cardiac interstitium.

Our *in situ* localization studies show expression of PDGF-C in

certain epithelial structures and of PDGFR-α in the adjacent mesenchyme, implying a potential for paracrine signalling in the developing embryo. PDGF-C expression seems to be particularly abundant at sites of ongoing ductal morphogenesis, which may also indicate a function in remodelling of connective tissue at these sites. The pattern of PDGF-C expression is distinct from those of PDGF-A and PDGF-B, indicating that these growth factors may have different roles despite their similar PDGFR-α-binding and signalling activities. This is illustrated in the mouse embryonic kidney, where PDGF-C is expressed in early aggregates of metanephric mesenchyme undergoing epithelial conversion, whereas PDGF-A is expressed in more mature tubular structures and PDGF-B is expressed by vascular endothelial cells. PDGFR-α is expressed in the mesenchyme of the kidney cortex, adjacent to sites of PDGF-C expression, indicating that the mesenchyme may be specifically targeted by PDGF-C. Indeed, mouse embryos lacking PDGFR-α show an extensive loss of the cortical mesenchyme adjacent to sites of PDGF-C expression which is not seen in mice lacking PDGF-A or PDGF-A and PDGF-B. This leads us to propose that PDGF-C has an essential function in the development of the kidney mesenchyme. Targeted mutagenesis of the PDGF-C gene will test this hypothesis. As discussed above, a further level of control of the biological activity of PDGF-C may be provided by extracellular proteolytic cleavage of PDGF-C. Clearly, the mechanism underlying the proteolytic activation of PDGF-C, as well as the nature and origin of the proteases involved, will provide important clues to the function of PDGF-C in interactions between the epithelium and the mesenchyme and in tissue remodelling in physiological and pathophysiological conditions. □

Methods

Isolation and analysis of human PDGF-C cDNA clones.

Two primers, 5′-GAAGTTGAGGAACCCAGTG (forward) and 5′-CTTGCCAAGAAGTTGCCAAG (reverse), derived from the identified human EST, were used to amplify a 368-base-pair (bp) fragment by PCR, using pfuge DNA from a human fetal lung 5′-STRETCH PLUS λgt10 cDNA library (Clontech) as a template. The fragment was cloned into the pCR2.1 vector (TA Cloning Kit, Invitrogen). The PCR fragment was labelled to high specific activity by random priming (Amersham) and the same cDNA library was screened by plaque hybridization to isolate full-length cDNA clones. Phage DNA was prepared from several of the identified clones, and a 3-kb insert from clone 11 was excised with *EcoRI* and subcloned into pBluescript (Stratagene). The nucleotide sequence was determined using internal and vector-specific primers.

Expression and analysis of PDGF-C in simian and insect cells.

The *EcoRI* fragment from full-length clone 11 was subcloned into the eukaryotic expression vector pSG5 (ref. 37). COS-1 cells were maintained in DMEM containing 10% FBS, 2 mM glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, and were transfected with the expression vector using diethylaminoethyl ether (DEAE)–dextran/chloroquine. Mock transfections with empty vectors served as negative controls. After 24h the medium was replaced by DMEM only. Serum-free medium from the cells was collected after an additional 24h and subjected to precipitation using ice-cold trichloroacetic acid (TCA). Precipitated proteins were washed several times with acetone to remove residual TCA and then subjected to SDS–PAGE under reducing conditions. PDGF-C was detected by immunoblotting using an antiserum raised against an internal peptide (see below) and bound antibodies were observed using enhanced chemiluminescence (ECL; Amersham).

The full-length coding part of the human PDGF-C cDNA (970bp) was amplified by PCR using Deep Vent DNA polymerase (New England Biolabs, Beverly, MA) under standard conditions. Primers used were: 5′-CGGGATCCCGAATCCAACCTGAGTAG (forward; includes *BamHI* site for in-frame cloning), and 5′-GGAATTCCTAATGGTGATGGTGATGATGTTTGTCATCGTCATCTCCTCC TGTGCTCCCTCT (reverse; includes *EcoRI* site and sequences encoding a C-terminal His$_6$ tag). The PDGFVVEGF domain of human PDGF-C (residues 230–363) was amplified by PCR as above. Primers used were 5′-CGGATCCCGAAGAAAATCCAGAGTGGTG (forward; includes *BamHI* site for in-frame cloning), and 5′-GGAATTCCTAATGGTGATGGTGATGATGTTTGTCATCGTCA TCTCCTCCTGTGCTCCCTCT (reverse; includes *EcoRI* site for cloning and sequences encoding a C-terminal His$_6$ tag preceded by an enterokinase site). Amplified products were digested with *BamHI* and *EcoRI* and cloned into the baculovirus expression vector pAcGP67A. Constructs were verified by nucleotide sequencing. Expression vectors were then co-transfected with BaculoGold linearized baculovirus DNA into Sf9 insect cells; generated baculoviruses were amplified (Pharmingen). Sf9 cells, adapted to serum-free medium, were infected with recombinant baculovirus at a multiplicity of infection of about 7. Media containing recombinant proteins were collected 4 days after infection and purified using Ni–NTA–agarose (Qiagen). Eluted proteins were analysed by SDS–PAGE using 11% polyacrylamide gels under reducing and non-reducing conditions, and stained with Coomassie brilliant blue. For immunoblotting analyses, recombinant proteins were observed using rabbit antibodies against an N-terminal peptide (residues 29–48, amino-acid sequence CKPQSSMKRQNCVQDPQHERC), or against an internal peptide (residues 230–250, amino-acid sequence CGRKSRVVDLNTLTEEVRLYSC). Both peptides contained extra cysteine residues to facilitate subsequent conjugation to the carrier protein. Anti-peptide antisera were generated in rabbits using peptides conjugated to keyhole-limpet haemocyanin by *N*-succinimidyl-3-(3-pyridyldithio)-propionate (SPDP, Pierce) as antigens.

Rabbit antisera against full-length PDGF-CC were generated by subcutaneous and intramuscular injections of the purified protein using 25–50μg protein per injection. Affinity-purified antibodies against the core domain of PDGF-CC were obtained by passing rabbit antisera over a column of the core domain of PDGF-CC coupled to CNBr-activated Sepharose 4B (Pharmacia). Bound antibodies were eluted with 0.3M glycine buffer containing 500 mM NaCl and dialysed against PBS.

Receptor binding and activation and mitogenic activity of PDGF-CC.

Competition-binding experiments were carried out essentially as described[38] using porcine aortic endothelial (PAE) cells with stable expression of human PDGFR-α and PDGFR-β. Indicated concentrations of human full-length and core-domain PDGF-CC or human PDGF-BB were mixed with 1ng ml^{-1} of^{125}I-labelled PDGF-BB in binding buffer (1mg ml^{-1} BSA in PBS). Aliquots were incubated with receptor-expressing PAE cells plated in 24-well culture dishes on ice for 90min. After three washes with binding buffer, ^{125}I-labelled PDGF-BB bound to cells was extracted by cell lysis in 20mM Tris–HCl pH 7.5, 10% glycerol and 1% Triton X-100. The amount of cell-bound radioactivity was determined using a γ-counter.

For direct-binding experiments, 10μg PDGF-CC core domain in 100mM sodium borate buffer, pH 8.5, was ^{125}I-labelled with Bolton–Hunter reagent (Amersham). The specific activity of the labelled protein was 4–8×10^6 c.p.m. per μg protein. Binding reactions to PAE cells expressing PDGFR-α or PDGFR-β and competition analyses were carried out essentially as above.

To monitor growth-factor-induced tyrosine phosphorylation of PDGFR-α, serum-starved PAE cells stably expressing human PDGFR-α were incubated on ice for 90min in PBS supplemented with 1mg ml^{-1} BSA and the indicated concentrations of PDGF-AA, full-length PDGF-CC, PDGF-CC core domain or a mixture of PDGF-AA and PDGF-CC core domain. Cells were lysed in 20mM Tris–HCl pH 7.5, 0.5% Triton X-100, 0.5% deoxycholic acid, 10mM EDTA, 1mM orthovanadate, 1 mM PMSF and 1% Trasylol. PDGFR-α was immunoprecipitated using a specific antiserum[39]. After separation by SDS–PAGE, precipitated receptors were transferred to nitrocellulose filters; filters were then probed with anti-phosphotyrosine antibody PY20 (Transduction Laboratories). After incubation with horseradish peroxidase (HRP)-conjugated anti-mouse antibodies, tyrosine-phosphorylated proteins were detected using ECL (Amersham).

Mitogenic activity of PDGF-CC was determined using a [^{3}H]thymidine-incorporation assay on AG1519 human-foreskin fibroblasts (Coriell Cell Repository, Camden, NJ). The assay was carried as described[9]. Serum-starved cells were incubated for 24h in serum-free medium supplemented with 1mg ml^{-1} BSA and the indicated concentrations of growth factors in the presence of 0.2μCi [^{3}H]thymidine. After TCA precipitation, incorporation of [^{3}H]thymidine into cellular DNA was determined using a β-counter.

Data from all receptor-binding assays and the proliferation assay are presented as means of triplicates from representative experiments. All assays, including the receptor-phosphorylation assay, were repeated at least three times with similar results.

Plasmin digestion of full-length PDGF-CC.

Full-length PDGF-CC was digested with plasmin in 20mM Tris–HCl pH 7.5, containing 1mM $CaCl_2$, 1 mM $MgCl_2$ and 0.01% Tween-20 for 0.5–2h at 37°C using 2–3 units of plasmin (Sigma) per ml. Progression of the digestion was analysed by SDS–PAGE under reducing conditions and immunoblotting using a rabbit antiserum against the internal peptide and affinity-purified antibodies against the PDGF-CC core domain (see above). Aliquots of digested and undigested samples were diluted in binding buffer and assayed for inhibition of binding of ^{125}I-labelled PDGF-CC core domain to PAE cells expressing PDGFR-α as described above.

Transgenic expression of full-length PDGF-C in mouse heart.

The sequence encoding the human c-Myc epitope[40] was introduced at the 5′ end of the coding region of mouse PDGF-C cDNA (to be reported elsewhere) by PCR mutagenesis with TaqVent polymerase (Biolabs) using the following primers: 5′-GGGAATTCTCAGCCAAATGCTCCTCTCGTC (forward), and 5′-GGATTCTTACAAGTCTCTTCAGAAATAAGCTTTTGTTCCCCTCCTGCGTTTCTCT (reverse). The resulting 1,300-bp fragment was subcloned into the pBluescript-based transgenic vector between the α5-Mb promoter for mouse α-myosin heavy chain and the 250-bp polyadenylation sequence from SV40 (ref. 19). The linearized and purified transgene fragment was injected into male pronuclei of fertilized mouse oocytes (Mouse Camp, Karolinska Institutet, Stockholm). Tail DNA prepared from the resulting pups was screened for the presence of the transgene by PCR using the mouse PDGF-C-specific primer 5′-GTGTCGATACGGGAAGAG (forward) and the human c-Myc-specific primer 5′-GTCTTCTCAGAAATAAGC (reverse). A 284-bp fragment was amplified in transgenic founders. Two transgenic lines were generated by backcrossing male founders to C57Bl females. At 6–8 weeks of age, transgenic animals and littermate controls were killed; hearts were removed, washed in ice-cold PBS and prepared for routine histological and biochemical analysis. Hearts were fixed in 4% paraformaldehyde in PBS overnight at 4°C, dehydrated, embedded in paraffin wax and sectioned. Tissue sections were stained in haematoxylin/eosin using standard techniques.

For analysis of PDGF-A expression, total cellular RNA (40μg) was prepared from normal and transgenic hearts using the guanidinium thiocyanate/acid phenol method[41] and stored at −80°C. The antisense PDGF-A probe was generated from a 900-bp subcloned *EcoRI* fragment of mouse PDGF-A cDNA using T7 RNA polymerase (Promega) and [^{32}P]UTP (Amersham). RNase protection analysis was used to quantify expression levels of PDGF-A in hearts of normal and PDGF-C-transgenic mice (Ambion, Austin, TX). The protected 320-bp band, corresponding to PDGF-A, was quantified by phosphorimaging and normalized against the 250-bp fragment corresponding to β-actin, the internal control (n = 3 for both normal and PDGF-C-transgenic hearts).

For biochemical analysis, hearts were minced and sonicated in ice-cold 10mM Tris–HCl buffer, pH 7.6, containing 0.1M NaCl, 1 mM EDTA, 1 μg ml^{-1} aprotinin and 4 mM phenylmethylsulphonyl fluoride. Supernatants were collected after centrifugation at 12,000*g* for 20min at 4°C; aliquots were then subjected to SDS–PAGE under reducing conditions. For immunoblotting analysis, an anti-c-Myc mouse monoclonal antibody (9E10) and a rabbit anti-mouse-PDGF-C antiserum were used. Bound antibodies were observed using ECL. Rabbit anti-mouse-PDGF-C antiserum was generated as described above using the peptide CVKKSRVVVNLNLKEEVKLYSC (residues 230–250 of mouse PDGF-C).

Northern blot analysis.

A commercial human multiple tissue northern blot (MTN, Clontech) was sequentially hybridized with different probes at high stringency using ExpressHyb hybridization solution (Clontech). The 348-bp ^{32}P-labelled PCR fragment from PDGF-C cDNA, generated and labelled as described above, was hybridized overnight; filters were washed as recommended and then exposed overnight on film at −70 °C. Expression of PDGF-A mRNA was analysed as described above using a full-length cDNA fragment as a probe[31].

In situ localization of PDGF-C transcripts in normal and mutant mice.

Non-radioactive *in situ* hybridization was carried out as described[4]. Mouse PDGF-C probes were derived from a 1.6-kb cDNA clone in pBluescript. Antisense full-length and internal 650-bp probes gave identical hybridization patterns. Hybridization patterns shown are for embryos at E16.5, but analogous patterns were seen at E14.5, E15.5 and E17.5. An internal 850-bp sense probe was used as a control and gave no consistent pattern of hybridization to sections.

Heterozygote mutants of PDGF-A, PDGF-B and PDGFR-α were bred as C57BL/129sv hybrids and intercrossed to produce homozygote mutant embryos. Compound PDGF-A/PDGF-B heterozygotes were crossed to generate double PDGF-A/B-knockout embryos. Because of the high degree of lethality of PDGF-A-knockout embryos before E10 (ref. 9), the proportion of double-knockout E16.5 embryos obtained in such crosses was less than 1 in 40. The histology of kidney phenotypes was verified on at least two embryos of each genotype.

RECEIVED 30 NOVEMBER 1999; REVISED 28 FEBRUARY 2000; ACCEPTED 15 MARCH 2000; PUBLISHED 7 APRIL 2000.

1. Raines, E. W., Bowen-Pope, D. F. & Ross, R. in *Handbook of Experimental Pharmacology: Peptide Growth Factors and their Receptors Vol. I* (eds Sporn, M. B. & Roberts, A. B.) 173–262 (Springer, New York, 1990).
2. Heldin, C. H., Ostman, A. & Rönnstrand, L. Signal transduction via platelet-derived growth factor receptors. *Biochim. Biophys. Acta* 1378, F79–F113 (1998).
3. Ataliotis, P. & Mercola, M. Distribution and functions of platelet-derived growth factors and their receptors during embryogenesis. *Int. Rev. Cytol.* 172, 95–127 (1997).
4. Betsholtz, C., Westermark, B., Ek, B. & Heldin, C.-H. Coexpression of a PDGF-like growth factor and PDGF receptors in a human osteosarcoma cell line: implications for autocrine receptor activation. *Cell* 39, 447–457 (1984).
5. Keating, M. T. & Williams, L. T. Autocrine stimulation of intracellular PDGF receptors in *v-sis* transformed cells. *Science* 239, 914–916 (1988).
6. Heldin, C. H. & Westermark, B. Mechanism of action and *in vivo* role of platelet-derived growth factor. *Physiol. Rev.* 79, 1283–1316 (1999).
7. Lindahl, P. & Betsholtz, C. Not all myofibroblasts are alike: revisiting the role of PDGF-A and PDGF-B using PDGF-targeted mice. *Curr. Opin. Nephrol. Hypertens.* 7, 21–26 (1998).
8. Lindahl, P. *et al.* Role of platelet-derived growth factors in angiogenesis and alveogenesis. *Curr. Top. Pathol.* 92, 27–33 (1999).
9. Boström, H. *et al.* PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* 85, 863–873 (1996).
10. Karlsson, L., Bondjers, C. & Betsholtz, C. Roles for PDGF-A and sonic hedgehog in development of mesenchymal components of the hair follicle. *Development* 126, 2611–2621 (1999).
11. Fruttiger, M. *et al.* Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. *Development* 126, 457–467 (1999).
12. Soriano, P. The PDGFα receptor is required for neural crest cell development and for normal patterning of the somites. *Development* 124, 2691–2700 (1997).
13. Leveen, P. *et al.* Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev.* 8, 1875–1887 (1994).
14. Soriano, P. Abnormal kidney development and hematological disorders in PDGFβ-receptor mutant mice. *Genes Dev.* 8, 1888–1896 (1994).
15. Lindahl, P. *et al.* Paracrine PDGF-B/PDGF-Rbeta signaling controls mesangial cell development in kidney glomeruli. *Development* 125, 3313–3322 (1998).
16. Lindahl, P., Johansson, B. R., Leveén, P. & Betsholtz, C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* 277, 242–245 (1997).
17. Bork, P. Complement component C1r/C1s, bone morphogenic protein 1 and *Xenopus laevis* developmentally regulated protein UVS.2 share common repeats. *FEBS Lett.* 283, 9–12 (1991).
18. Ostman, A. Expression of three recombinant homodimeric isoforms of PDGF in *Saccharomyces cerevisiae*: evidence for difference in receptor binding and functional activities. *Growth Factors* 1, 271–281 (1989).
19. Subramaniam, A. *et al.* Tissue-specific regulation of the alpha-myosin heavy chain gene promoter in transgenic mice. *J. Biol. Chem.* 266, 24613–24620 (1991).
20. Simm, A., Nestler, M. & Hoppe, V. Mitogenic effect of PDGF-AA on cardiac fibroblasts. *Basic Res. Cardiol.* 93, Suppl 3, 40–43 (1998).
21. Vainio, S. & Muller, U. Inductive tissue interactions, cell signaling, and the control of kidney organogenesis. *Cell* 90, 975–978 (1997).
22. Oefner, C., D'Arcy, A., Winkler, F.K., Eggimann, B. & Hosang, M. Crystal structure of human platelet-derived growth factor BB. *EMBO J.* 11, 3921–3926 (1992).
23. Östman, A., Thyberg, J., Westermark, B. & Heldin, C.-H. PDGF-AA and PDGF-BB biosynthesis: proprotein processing in the Golgi complex and lysosomal degradation of PDGF-BB retained intracellularly. *J. Cell Biol.* 118, 509–519 (1992).
24. Nakayama, K. Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. *Biochem. J.* 327, 625–635 (1997).
25. Bork, P. & Beckmann, G. The CUB domain. A widespread module in developmentally regulated proteins. *J. Mol. Biol.* 231, 539–545 (1993).
26. Mineitup, S. K. *et al.* The intrinsic factor-vitamin B12 receptor and target of teratogenic antibodies is a megalin-binding peripheral membrane protein with homology to developmental proteins. *J. Biol. Chem.* 273, 5235–5242 (1998).
27. Nakamura, F., Tanaka, M., Takahashi, T., Kalb, R. G. & Strittmatter, S. M. Neuropilin-1 extracellular domains mediate semaphorin D/III-induced growth cone collapse. *Neuron* 21, 1093–1100 (1998).
28. Giger, R.J. *et al.* Neuropilin-2 is a receptor for semaphorin IV: insight into the structural basis of receptor function and specificity. *Neuron* 21, 1079–1092 (1998).
29. Kataharan, M. *et al.* Molecular dissection of the intrinsic factor-vitamin B12 receptor, cubilin, discloses regions important for membrane association and ligand binding. *J. Biol. Chem.* 274, 20540–20544 (1999).
30. Thielens, N. M., Bersch, B., Hernandez, J. F. & Arlaud, G. J. Structure and functions of the interaction domains of C1r and C1s: keystones of the architecture of the C1 complex. *Immunopharmacology* 42, 3–13 (1999).
31. Andersson, M., Östman, A., Westermark, B. & Heldin, C.-H. Characterization of the retention motif in the C-terminal part of the long splice form of platelet-derived growth factor A-chain. *J. Biol. Chem.* 269, 926–930 (1994).
32. Pekny, M. *et al.* Differences in binding to the solid substratum and extracellular matrix may explain isoform-specific paracrine effects of platelet-derived growth factor. *Growth Factors* 10, 77–87 (1994).
33. Park, I. E., Keller, G. A. & Ferrara, N. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol. Biol. Cell* 4, 1317–1326 (1993).
34. Hauser, S. & Weich, H. A heparin-binding form of placenta growth factor (PlGF-2) is expressed in human umbilical vein endothelial cells and in placenta. *Growth Factors* 9, 259–268 (1993).
35. Olofsson, B. *et al.* Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc. Natl Acad. Sci. USA* 93, 2576–2581 (1996).
36. Eriksson, U. & Alitalo, K. Structure, expression and receptor-binding properties of novel vascular endothelial growth factors. *Curr. Top. Microbiol. Immunol.* 237, 41–57 (1999).
37. Green, S., Issemann, I. & Sheer, E. A versatile *in vivo* and *in vitro* eucaryotic expression vector for protein engineering. *Nucleic Acid Res.* 16, 369 (1988).
38. Heldin, C.-H. *et al.* Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. *EMBO J.* 7, 1387–1393 (1988).
39. Eriksson, A., Siegbahn, A., Westermark, B., Heldin, C.-H. & Claesson-Welsh, L. PDGFα- and β-receptors activate unique and common signal transduction pathways. *EMBO J.* 11, 543–550 (1992).
40. Mori, S., Claesson-Welsh, L. & Heldin, C. H. Identification of a hydrophobic region in the cytosolic terminus of the platelet- derived growth factor beta-receptor which is important for ligand-mediated endocytosis. *J. Biol. Chem.* 266, 21158–21164 (1991).
41. Evan, G. I., Lewis, G. K., Ramsay, G. & Bishop, J. M. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell Biol.* 5, 3610–3616 (1985).
42. Chomczynski, P. & Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159 (1987).
43. Betsholtz, C. *et al.* cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumour cell lines. *Nature* 320, 695–699 (1986).

ACKNOWLEDGEMENTS

We thank B. Åkerblom and C. Raynoschek for technical assistance, and U. Engström for synthesizing peptides. This study was supported by grants from the Swedish and Finnish Cancer Societies, the Swedish Medical Research Council, the Göran Gustafsson Foundation, the Inga-Britt and Arne Lundberg Foundation, the Swedish Heart and Lung Foundation and the Novo Nordisk Foundation. The cooperative research agreement between the Ludwig Institute for Cancer Research, Stockholm branch, and Fuji Photo Film (Europe) is gratefully acknowledged.

Correspondence and requests for materials should be addressed to U.E. The nucleotide and amino-acid sequences of human PDGF-C have been deposited at GenBank under accession number AF244813.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2005 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 280, No. 29, Issue of July 22, pp. 26856–26862, 2005
Printed in U.S.A.

Structural Requirements for Activation of Latent Platelet-derived Growth Factor CC by Tissue Plasminogen Activator*

Received for publication, March 28, 2005, and in revised form, May 17, 2005
Published, JBC Papers in Press, May 23, 2005, DOI 10.1074/jbc.M503388200

Linda Fredriksson, Monika Ehnman, Christina Fieber, and Ulf Eriksson‡

From the Ludwig Institute for Cancer Research, Stockholm Branch, Box 240, S-171 77 Stockholm, Sweden

Platelet-derived growth factor C (PDGF-C) is one of four members in the PDGF family of growth factors, which are known mitogens and survival factors for cells of mesenchymal origin. PDGF-C has a unique two-domain structure consisting of an N-terminal CUB and a conserved C-terminal growth factor domain that are separated by a hinge region. PDGF-C is secreted as a latent dimeric factor (PDGF-CC), which undergoes extracellular removal of the CUB domains to become a PDGF receptor α agonist. Recently, the multidomain serine protease tissue plasminogen activator (tPA), a thrombolytic agent used for treatment of acute ischemic stroke, was shown to cleave and activate PDGF-CC. In this study we determine the molecular mechanism of tPA-mediated activation of PDGF-CC. Using various PDGF-CC and tPA mutants, we were able to demonstrate that both the CUB and the growth factor domains of PDGF-C, as well as the kringle-2 domain of tPA, are required for the interaction and cleavage to occur. We also show that Arg^{231} in PDGF-C is essential for tPA-mediated proteolysis and that the released "free" CUB domain of PDGF-C can act as a competitive inhibitor of the cleavage reaction. Furthermore, we studied how the PDGF-C/tPA axis is regulated in primary fibroblasts and found that PDGF-C expression is down-regulated by hypoxia but induced by transforming growth factor (TGF)-β_1 treatment. Elucidating the regulation and the mechanism of tPA-mediated activation of PDGF-CC will advance our knowledge of the physiological function of PDGF-CC and tPA and may provide new therapeutic opportunities for thrombolytic and cardiovascular therapies.

Platelet-derived growth factor C (PDGF-C)[1] was discovered a few years ago as the third member of the well characterized PDGF family of growth factors (1). The classical members of this family, PDGF-A and PDGF-B, have been intensively studied and are known to be important for connective tissue growth and maintenance, and overexpression has been observed in several pathological conditions, including malignancies and atherosclerosis (2). Since its discovery, PDGF-C has been shown to play a role in palate formation (3), fibrotic disease development (4, 5), and angiogenesis (6, 7). Recently a fourth member, PDGF-D, has been added to this family of growth factors (8, 9). The four PDGF chains assemble into five dimeric isoforms, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD, that exert their effects on cells through differential signaling via two known tyrosine kinase receptors, platelet-derived growth factor receptor (PDGFR)-α and PDGFR-β (10).

Unlike the classical members, PDGF-C and PDGF-D have a unique two-domain structure, with a so-called CUB domain N-terminal of the conserved growth factor domain (1, 8, 9). In order for the novel PDGFs to bind and activate the PDGFRs, the N-terminal CUB domains have to be removed through limited proteolysis by extracellular proteases. The origin of the protease involved in the activation of PDGF-DD still remains elusive, whereas the extracellular fibrinolytic protease tissue plasminogen activator (tPA) has been shown to be a potent activator of PDGF-CC (11).

tPA is a highly specific serine protease that consists of five structural domains, a finger domain, an epidermal growth factor-like domain, two kringle domains, and a trypsin-like protease domain (12). It is best known for its role in vascular fibrinolysis where it converts the zymogen plasminogen into plasmin, which in turn degrades the fibrin network in blood clots. The observation that tPA binds to fibrin via its finger and kringle-2 domains (13, 14), thus facilitating a localized generation of plasmin, has focused much attention on the use of tPA as a thrombolytic agent. In fact, tPA is currently used to treat acute myocardial infarction and is also approved for treatment of acute ischemic stroke (15). However, emerging evidence points at non-fibrinolytic functions of tPA, at least within the central nervous system, promoting events associated with synaptic plasticity and regulation of neurovascular permeability (16–18). Some of these studies claim the effect to be mediated by plasmin, whereas others show the effect to be independent of plasminogen activation (reviewed in Ref. 19). At present there are only two non-plasminogen substrates reported for tPA, namely PDGF-CC and the NR1 subunit of the *N*-methyl-D-aspartate receptor (11, 20).

A PDGF-CC/tPA stimulatory loop has recently been described to influence the growth of primary fibroblasts, which might have implications in the recruitment and growth of stromal fibroblasts into tumors and in wound-healing processes (11). It has also been shown that PDGF-CC can enhance delayed wound healing in diabetic mice (21) and revascularization of ischemic tissues (6), further emphasizing the therapeutic potentials of PDGF-CC. Clearly, it is of importance to determine the structural and regulatory requirements of PDGF-CC activation. Here, we describe the molecular mechanism of tPA-mediated cleavage of PDGF-CC. We demonstrate that both the CUB and the growth factor domains of PDGF-C

* This work was supported by grants from the Novo Nordisk Foundation, The Swedish Research Council, and Karolinska Institutet. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 46-8-52487109; Fax: 46-8-332812; E-mail: ulf.eriksson@ljcr.ki.se.

[1] The abbreviations used are: PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; DMEM, Dulbecco's modified Eagle's medium; PAE, porcine aortic endothelial; Ni-NTA, nickel-nitrilotriacetic acid; pNA, paranitroanilide; PAI, plasminogen activator inhibitor; FGF, fibroblast growth factor; TGF, transforming growth factor; tPA, tissue plasminogen activator.

TABLE I
Construct nomenclature and description of primers used

Construct	Description	Oligonucleotides
PC_{A150}	PDGF-CC truncation mutants	Sense: 5′-CCCAAGCTTCCTTCTGAACCAGGG-3′
PC_{A170}		Sense: 5′-CCCAAGCTTGTGAGTCCTTCAGTG-3′
PC_{A193}		Sense: 5′-CCCAAGCTTACTGCCTTTAGTACC-3′
PC_{A218}		Sense: 5′-CCCAAGCTTGACTTAGAAGATC-3′
PC_{A230}		Sense: 5′-CCCAAGCTTAGAAAATCCAGAGTG-3′
		Antisense: 5′-GGAATTCCTCCTGTGCTCCCTCTG-3′
$PD_{CUB}PC$	CUB region of PDGF-DD	Sense: 5′-GCGGATCCTCCCAAATGCACCGGCTC-3′
	Core region of PDGF-CC	Antisense: 5′-GCGAATTCATCTTCCAGCAAAGAATA-3′
		Sense: 5′-GCGAATTCACAGAAGCTGTGA-3′
		Antisense: 5′-GCGGATCCAGAATCAGCCACTGCACT-3′
$PC_{CUB}PD$	CUB region of PDGF-CC	Sense: 5′-GCTGGATCCAGACAGGGGACTCAGGCGAAT-3′
	Core region of PDGF-DD (including a His_6 encoding sequence)	Antisense: 5′-GCTACCGGTCCAGATCCACCACTCTGGA-3′
		Sense: 5′-CGTACCGGTTGAATGATGATGCCAAGCGT-3′
		Antisense: 5′-GCTCGAATTCTTAATGGTGATGGTGATGATG TCGAGGTGGTCTTGA-3′
PC_{CUB}	CUB domain of PDGF-CC (including a human c-*myc* encoding sequence)	Sense: 5′-GCGAATTCTGAGCTCTCACCCCAGTC-3′
		Antisense: 5′-GCGGATCCTTACAAGTCTTCTTCAGAAATAAG CTTTTGTTCTGGCATGACAATGTT-3′
PC_{AAAA}	PDGF-CC cleavage site mutants	Antisense: 5′-TCCGTTAACAAGTTCAGATCCACCACGGCGGCG GCGGCTCCAAAAAC-3′
PC_{R231A}		Antisense: 5′-TCCGTTAACAAGTTCAGATCCACCACTCTGGATT TGGCTCCAAAAAC-3′
PC_{K232A}		Antisense: 5′-TCCGTTAACAAGTTCAGATCCACCACTCTGGAG GCTCTTCCAAA-3′
PC_{R234A}		Antisense: 5′-TCCGTTAACAAGTTCAGATCCACCACGGCGGAT TTTCTTCCAAA-3′
		Sense 5′-TCCGTTAACAGAGGAGGTAAGATTATACAGCTGC ACACCTCGTAAC-3′
tPA_{wt}	tPA truncation mutants (cloning into pSecTag2B)	Sense: 5′-CGTGGATCCAGCCAGGAAATCCATGCC-3′
tPA_{A70}		Sense: 5′-CGTGGATCCCACTCAGTGCCTGTCAAA-3′
tPA_{A119}		Sense: 5′-CGTGGATCCGAAATAGATACCAGGGGC-3′
tPA_{A200}		Sense: 5′-CGTGGATCCTCTGAGGGAAACAGTGAC-3′
tPA_{A230}		Sense: 5′-CGTGGATCCGGTCTACGACAGTACAGC-3′
		Antisense: 5′-ATGCCTCGAGCCGGTCGCATGTTGTCACG-3′
tPA_{ss}	tPA truncation mutants (subcloning into pcDNA3.1/Zeo(−))	Sense: 5′-ATTCTAGAACCCAAGCTGGCTAGCCA-3′
		Antisense: 5′-ATTCTAGATTACAGATCCTCTTCTGAGATG-3′

and the kringle-2 domain of tPA are necessary for interaction of the two proteins and that Arg^{231} in the hinge region of PDGF-CC is needed for cleavage by tPA.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-1 cells and primary fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, and porcine aortic endothelial (PAE) cells were kept in supplemented F12 medium. The cells were cultured at 37 °C in a humidified 5% O_2 atmosphere. Kidney primary fibroblast cultures were prepared as described previously, and experiments were performed on cells at passages 4–8 (11).

Plasmid Construction—The nucleotide sequences encoding the various PDGF-C and tPA truncation mutants, the CUB chimeric constructs ($PD_{CUB}PC$ and $PC_{CUB}PD$), the CUB domain of PDGF-C (PC_{CUB}), and the cleavage site mutants were amplified by PCR using gene-specific primers (see Table I) and *Taq* DNA polymerase (Invitrogen) if not stated otherwise. The PCR fragments of the PDGF-C and tPA truncation mutants, as well as the full-length tPA (tPA_{wt}) lacking the signal sequence (used as control), were cloned in-frame with the signal sequence of the eukaryotic expression vector pSecTag2B (Invitrogen). The tPA truncation mutants and tPA_{ss} were then subcloned into the expression vector pcDNA3.1/Zeo(−) (Invitrogen) accompanied by the Igκ-chain leader sequence and *c-myc* epitope, but excluding the His_6 tag, from pSecTag2B. The amplified $PD_{CUB}PC$ and $PC_{CUB}PD$ fragments of the CUB regions (residues 1–172 of PDGF-D and 1–238 of PDGF-C, respectively) and the growth factor regions (residues 166–345 of PDGF-C and 261–370 of PDGF-D, respectively) were ligated and cloned into the eukaryotic expression vectors pSG5 ($PD_{CUB}PC$) (22) or a modified pSecTag2A ($PC_{CUB}PD$; part of the multiple cloning site between SfiI and KpnI was removed by restriction; Invitrogen). The PC_{CUB} PCR products (residues 1–165 of PDGF-C) was directionally cloned into pSG5. To generate the PDGF-C cleavage site mutants, primers were designed to enable PCR amplification of the entire vector template, human PDGF-C in pSG5 (1), using Phusion DNA polymerase (Finnzymes). Point mutations and a HpaI site for in-frame cloning were included in the primer sequences. The 7.1-kb PCR products were cleaved and ligated. All primers used were purchased from Invitrogen, and all of the constructs were verified by nucleotide sequencing.

Transfection, Immunoblotting, and Receptor Activation—Subconfluent COS-1 cells were transfected with the various expression constructs using Lipofectamine Plus reagent in serum-free DMEM (Invitrogen). Transfection with empty vectors served as negative control (mock). After 4 h the transfection medium was replaced by supplemented DMEM overnight and thereafter by DMEM only. The conditioned serum-free medium was collected 48 h after transfection and used in receptor stimulation studies. Alternatively the proteins were precipitated using trichloroacetic acid as described previously (1). All precipitates were subjected to SDS-PAGE under reducing conditions, immunoblotted, and visualized by enhanced chemiluminescence plus reagent (ECL+, Amersham Biosciences). PDGF-C species and PC_{CUB}PD were detected by immunoblotting using affinity-purified polyclonal rabbit antibodies against PDGF-C (1) and PDGF-D (8), respectively. tPA was detected using sheep polyclonal antibodies against human tPA (ab9030, Abcam) and tPA truncation mutants using rabbit polyclonal antibodies against human *c-myc* (sc-789, Santa Cruz Biotechnology).

To monitor growth factor-induced tyrosine phosphorylation of PDGFR-α and PDGFR-β, serum-starved PAE cells stably expressing the respective human PDGFRs, were incubated for 90 min on ice with conditioned medium from transfected COS-1 cells. PAE cells treated with either conditioned medium from COS-1 cells transfected with empty vector (mock) or with recombinant PDGF-BB (100 ng/ml), or alternatively the recombinant growth factor domain of PDGF-CC (100

ng/ml), were used as controls. Following treatment the PAE cells were lysed in 20 mM Tris-HCl pH, 7.5, 0.5% Triton X-100, 0.5% deoxycholic acid, 150 mM NaCl, 5 mM EDTA, 200 μM orthovanadate, and complete protease inhibitor mixture, and the PDGFRs were immunoprecipitated using specific antisera (23). Precipitated receptors were separated by SDS-PAGE under reducing conditions. Tyrosine-phosphorylated receptors were detected by immunoblotting using an anti-phosphotyrosine antibody (PY99, Santa Cruz Biotechnology). Bound antibodies were visualized as above.

Protein-Protein Interaction Studies—To determine which domain(s) of tPA and PDGF-CC are involved in the protein-protein interaction between the two proteins, His_6-tagged recombinant PDGF-CC protein species, expressed using the baculovirus expression system as described previously (1), were bound to nickel-nitrilotriacetic acid (Ni-NTA)-agarose (Qiagen) and then incubated for 90 min at room temperature with conditioned serum-free media from COS-1 cells transfected with the tPA truncation mutants. Uncoated Ni-NTA beads were used as negative control. The beads were thoroughly washed, and the His_6-tagged PDGF-CC species were specifically eluted with 400 mM imidazole (Sigma). Eluted proteins were analyzed by SDS-PAGE under reducing conditions and immunoblotted with rabbit polyclonal antibodies against human *c-myc* (see above) to detect co-eluted tPA truncation species. The membranes were subsequently stripped and reprobed with PDGF-C-specific antibodies to detect input of full-length and core PDGF-C species or an anti-His monoclonal antibody (C-terminal, Invitrogen) to detect input of CUB protein. Bound antibodies were detected as described above.

Chromogenic Assay—To confirm functional protease activity among the tPA truncation mutants, a chromogenic assay was developed. Conditioned serum-free media from COS-1 cells transfected with the tPA truncation constructs were subjected to size-exclusion chromatography using NAP-10 columns (Amersham Biosciences) to enable buffer exchange to Tris-buffered saline. The protease activity analysis was performed in flat-bottomed microplates with 0.2 mM Spectrozyme tPA (American Diagnostica) as a chromogenic substrate for tPA. The formation of paranitroanilide (pNA), *i.e.* the amount of cleaved substrate, was measured photometrically at 405 nm. Comparable product amounts suggest functional protease activity. Buffer-exchanged conditioned media from mock-transfected cells were used as negative control. Purified human tPA was used to define maximal activity (T7776, Sigma).

Regulation of PDGF-C, tPA, and Plasminogen Activator Inhibitor (PAI)-1 Expression—To determine how PDGF-C, tPA, and its inhibitor PAI-1 are regulated by various growth factors and metabolic conditions, primary kidney fibroblasts were plated at subconfluence in 6-well plates. Following attachment, the medium was exchanged for serum-free DMEM in the absence or presence of TGF-β, (5 ng/ml), fibroblast growth factor (FGF)-2 (5 ng/ml, R&D Systems), or high glucose (30 mM); alternatively the cells were placed in hypoxic conditions (1% oxygen). After 24 h the conditioned serum-free media were collected, and the proteins were trichloroacetic acid-precipitated and subjected to SDS-PAGE followed by immunoblotting. PDGF-C and tPA were detected using specific antibodies (see above), and PAI-1 was detected using a rabbit anti-PAI antibody (sc-8979, Santa Cruz Biotechnology).

RESULTS

tPA-mediated Proteolysis Depends on Both Structural Domains of PDGF-CC—We mapped the structural requirements for recognition of latent PDGF-CC as a substrate for tPA using mutated forms of PDGF-CC in a co-transfection assay. The mutants of PDGF-CC included chimeric forms of PDGF-C, one carrying the CUB domain of PDGF-D and the hinge region and growth factor domain of PDGF-C ($PD_{CUB}PC$) and the other one carrying the CUB domain and the hinge region of PDGF-C and the growth factor domain of PDGF-D ($PC_{CUB}PD$) (schematically illustrated in Fig. 1*A*). In addition, a truncation mutant lacking the CUB domain of PDGF-C was also employed ($PC_{\Delta 150}$). All mutants were properly expressed in transfected COS-1 cells, formed disulfide-linked dimers (data not shown), and were efficiently secreted in the conditioned medium (Fig. 1*B*). When co-transfected with tPA, the generation of a 22-kDa protected fragment from $PD_{CUB}PC$ was significantly reduced as compared with wild-type PDGF-CC (PC_{WT}), whereas no cleavage product was detected in co-transfections with $PC_{CUB}PD$ or with $PC_{\Delta 150}$ (Fig. 1*B*).

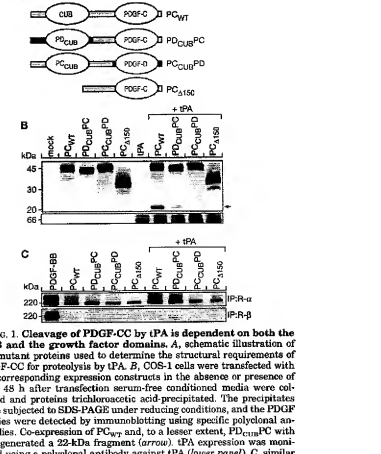

FIG. 1. **Cleavage of PDGF-CC by tPA is dependent on both the CUB and the growth factor domains.** *A*, schematic illustration of the mutant proteins used to determine the structural requirements of PDGF-CC for proteolysis by tPA. *B*, COS-1 cells were transfected with the corresponding expression constructs in the absence or presence of tPA. 48 h after transfection serum-free conditioned media were collected and proteins trichloroacetic acid-precipitated. The precipitates were subjected to SDS-PAGE under reducing conditions, and the PDGF species were detected by immunoblotting using specific polyclonal antibodies. Co-expression of PC_{WT} and, to a lesser extent, $PD_{CUB}PC$ with tPA generated a 22-kDa fragment (*arrow*). tPA expression was monitored using a polyclonal antibody against tPA (*lower panel*). *C*, similar results were obtained when conditioned serum-free media from transfected COS-1 cells were used to study induction of tyrosine phosphorylation of either PDGFR-α (*R-α, upper panel*) or PDGFR-β (*R-β, lower panel*) expressed in PAE cells. PAE cells were stimulated with the conditioned media on ice and lysed, and then the PDGFRs were immunoprecipitated (*IP*) using specific antibodies. Phosphorylated receptors were detected by immunoblot analysis using an anti-phosphotyrosine antibody. Recombinant PDGF-BB (100 ng/ml) was used as positive control.

These results were verified in receptor stimulation experiments where conditioned media from transfected COS-1 cells were applied onto PAE cells with stable expression of PDGFR-α (*upper panel*) or PDGFR-β (*lower panel*), respectively (Fig. 1*C*). Following immunoprecipitation of the respective receptors, stimulation was measured as induction of receptor tyrosine phosphorylation. As shown previously, media from COS-1 cells co-expressing tPA and wild-type PDGF-CC induced strong PDGFR-α activation comparable with PDGF-BB-stimulated controls (11), whereas media from COS-1 cells co-expressing tPA with $PD_{CUB}PC$ induced weaker PDGFR-α activation. Cells stimulated with conditioned media expressing the PDGF-CC mutants alone or co-expressing tPA with $PC_{CUB}PD$ or the truncation mutant $PC_{\Delta 150}$, respectively, showed only background levels of PDGFR-α activation. As cleavage of $PC_{CUB}PD$ would release the growth factor domain of PDGF-DD, a PDGFR-β agonist, the conditioned media were also applied to PDGFR-β expressing PAE cells, but none induced PDGFR-β stimulation. These findings indicate that both the CUB and the growth factor domains are necessary for efficient proteolytic cleavage of latent PDGF-CC by tPA.

The Majority of the Hinge Region Is Removed in Active PDGF-CC—The finding that the truncation mutant $PC_{\Delta 150}$

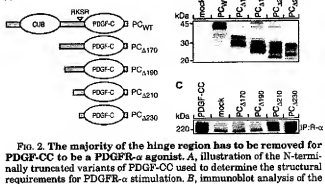

FIG. 2. **The majority of the hinge region has to be removed for PDGF-CC to be a PDGFR-α agonist.** *A*, illustration of the *N*-terminally truncated variants of PDGF-CC used to determine the structural requirements for PDGFR-α stimulation. *B*, immunoblot analysis of the truncation mutants in conditioned serum-free medium collected from transfected COS-1 cells. Equivalent expression of the PDGF-C species was detected using specific polyclonal antibodies. *C*, induction of tyrosine phosphorylation of PDGFR-α expressed in PAE cells. Conditioned serum-free media from transfected COS-1 cells were applied to the PAE cells, and the PDGFR-α (*R-α*) was immunoprecipitated (*IP*). Only the two shortest mutants induced efficient phosphorylation comparable with the positive control recombinant PDGF-CC (100 ng/ml, growth factor domain of PDGF-CC).

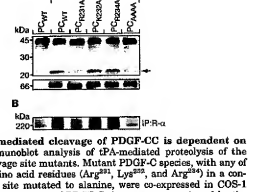

FIG. 3. **tPA-mediated cleavage of PDGF-CC is dependent on Arg^{231}.** *A*, immunoblot analysis of tPA-mediated proteolysis of the PDGF-CC cleavage site mutants. Mutant PDGF-C species, with any of three basic amino acid residues (Arg^{231}, Lys^{232}, and Arg^{234}) in a conserved tribasic site mutated to alanine, were co-expressed in COS-1 cells with tPA. The extent of PDGF-C cleavage was monitored by the presence of the 22-kDa band in immunoblots using PDGF-C-specific antibodies (*arrow, upper panel*). Cleavage was completely abolished in the PC_{R231A} mutant suggesting that Arg^{231} is essential for cleavage to occur. A mutant with the entire region Arg^{231}–Arg^{234} replaced by alanine residues, PC_{AAAA}, was used as a cleavage-resistant control, whereas wild-type PDGF-CC (PC_{WT}) was used as a positive control. tPA expression was monitored using specific polyclonal antibodies (*lower panel*). *B*, receptor stimulation studies, measured as induction of tyrosine phosphorylation of PDGFR-α. Following stimulation, PDGFR-α (*R-α*) was immunoprecipitated (*IP*) using specific receptor antibodies, and phosphorylated receptors were detected by immunoblot analysis using an anti-phosphotyrosine antibody. Conditioned serum-free media from COS-1 cells co-transfected with tPA and either PC_{R231A} or PC_{AAAA} failed to induce efficient phosphorylation of the receptor, thus confirming the results seen in *A*.

failed to induce PDGFR-α activation indicates that not only the CUB domain but also parts of the hinge region have to be removed for receptor activation. To understand the structural requirements of PDGF-CC for receptor binding and activation, a series of truncated mutants of PDGF-C, lacking the CUB domain and increasing portions of the hinge region, was developed (schematically illustrated in Fig. 2*A*). All mutants formed disulfide-linked dimers (data not shown) and were efficiently secreted in the conditioned medium by transfected COS-1 cells (Fig. 2*B*). The multiple species of the truncated mutants seen in the immunoblots are possibly due to exposure and subsequent glycosylation of the putative *N*-glycosylation site present in the growth factor domain of PDGF-C (1). However, it cannot be ruled out that removal of the CUB domain leaves the otherwise protected hinge region vulnerable for degradation.

The truncation mutants were analyzed for their ability to activate PDGFR-α in PAE cells. Conditioned media containing equal amounts of the truncated mutant proteins of PDGF-CC (determined by enzyme-linked immunosorbent assay) were applied onto PAE cells, and the activation of PDGFR-α was monitored by induction of receptor tyrosine phosphorylation (Fig. 2*C*). The results showed that the two shortest mutants, $PC_{\Delta 210}$ and $PC_{\Delta 230}$, efficiently activated PDGFR-α, whereas mutants with additional parts of the hinge region, separating the CUB and the growth factor domains in PDGF-C, failed to do so. Thus, in order for PDGF-CC to be a receptor agonist, both the CUB domain and the majority of the hinge region have to be removed from the growth factor, allowing at most the last 40 amino acids of the hinge region to remain, indicating that the cleavage site resides in this region.

Arg^{231} in the Hinge Region of PDGF-CC Is Essential for tPA-mediated Cleavage—Within this stretch of amino acids we have previously identified a putative tribasic processing site (amino acid residues -Arg^{231}-Lys^{232}-Ser^{233}-Arg^{234}- in human PDGF-C) based on comparison with the well known processing sites in PDGF-A and PDGF-B (1). Recently we reported tPA to cleave mouse PDGF-CC in, or at least around, this conserved site (11). To better characterize which of the three basic amino acids, Arg^{231}, Lys^{232}, and Arg^{234}, is important for cleavage to occur, the amino acids were individually mutated to alanine residues. The expression constructs encoding these PDGF-C mutants were separately co-transfected with tPA. The extent of PDGF-CC cleavage was monitored by immunoblotting as the presence of the 22-kDa band (Fig. 3*A*) and induction of PDGFR-α phosphorylation (Fig. 3*B*). Wild-type PDGF-C and a mutant resistant to tPA-mediated cleavage with the entire region Arg^{231}–Arg^{234} replaced by alanine residues were used as controls. These experiments demonstrate that the cleavage site for tPA is confined to the Arg^{231}–Arg^{234} segment in human PDGF-CC and that Arg^{231} is essential for cleavage to occur, whereas the other basic amino acid residues in the site are less important.

The CUB Domain of PDGF-C Acts as a Specific Inhibitor of tPA-mediated Cleavage—Based on our previous findings that the CUB domain of PDGF-C, but not that of PDGF-D, specifically interacts with tPA (11) and the above results showing that the CUB domain is necessary for specific cleavage, we hypothesized that the released free CUB domain of PDGF-C might act as a competitive inhibitor of tPA-mediated activation of PDGF-CC. To test this hypothesis we co-transfected COS-1 cells with wild-type PDGF-CC and tPA in the absence or presence of an expression construct expressing the free CUB domain of PDGF-C (PC_{CUB}). We were able to show that the CUB domain of PDGF-C efficiently competed for the processing of latent PDGF-CC by tPA, as determined by immunoblot experiments (Fig. 4*A*), and activation, as determined by induction of PDGFR-α phosphorylation (Fig. 4*B*), thus suggesting that the CUB domain may indeed act as a competitive inhibitor of tPA-mediated proteolysis.

Kringle-2 of tPA Is Necessary for Cleavage of PDGF-CC—To determine which of the structural domains of tPA is necessary for efficient cleavage of latent PDGF-CC we created truncated forms of tPA (schematically illustrated in Fig. 5*A*) and expressed them in transfected COS-1 cells (Fig. 5*B*). To ensure that the mutated tPA proteins were functionally active, a tPA Spectrozyme substrate was added to buffer-exchanged conditioned serum-free media from transfected COS-1 cells, and after 3 h the formation of *p*NA, indicative of the amount of cleaved tPA Spectrozyme substrate, was measured photometrically (Fig. 5*C*). All tPA mutants induced *p*NA formation in a similar fashion suggesting correct protein folding and intact protease activity. Purified tPA was used to define maximal

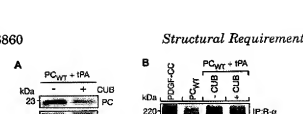

FIG. 4. **The CUB domain of PDGF-C can inhibit tPA-mediated cleavage of PDGF-CC.** *A*, COS-1 cells were co-transfected with expression constructs of wild-type PDGF-C (PC_{WT}) and tPA in the absence or presence of an expression construct expressing the *c-myc*-tagged free CUB domain of PDGF-C (PC_{CUB}). Trichloroacetic acid-precipitated proteins from conditioned serum-free media were immunoblotted using polyclonal antibodies against PDGF-C, tPA, and the human *c-myc* epitope, respectively. Co-expression of the free CUB domain of PDGF-C with PC_{WT} and tPA in COS-1 cells markedly reduced the tPA-mediated cleavage of PDGF-CC monitored as the presence of the 22-kDa band. *B*, induction of tyrosine phosphorylation of PDGFR-α expressed in PAE cells. Conditioned serum-free media from transfected COS-1 cells were applied to the PAE cells, and the PDGFR-α (*R-α*) was immunoprecipitated (*IP*). The presence of free CUB reduced phosphorylation of PDGFR-α as compared with when CUB was not co-expressed with PDGF-CC and tPA. Recombinant PDGF-CC (100 *ng*/ml, growth factor domain of PDGF-CC) and PC_{WT} alone were used as controls.

activity, and within 24 h all mutants had induced maximal formation of pNA. Buffer-exchanged conditioned medium from cells transfected with empty vector (mock) was used as negative control.

To assess the structural requirements of tPA for cleavage of PDGF-CC, the tPA truncation mutants were co-expressed in COS-1 cells together with wild-type PDGF-CC, and cleavage was determined by the formation of the 22-kDa protected fragment of PDGF-C. Our results show that co-transfection of the shortest tPA mutant, $tPA_{\Delta 300}$, containing only the trypsin-like protease domain, with PDGF-CC significantly reduced the generation of the 22-kDa band as compared with any of the other tPA mutants (Fig. 5*D*). These data suggest that, although $tPA_{\Delta 300}$ is expressed and functional, the kringle-2 domain is required for tPA to efficiently cleave PDGF-CC.

Interaction between tPA and PDGF-CC Is Mediated by kringle-2 in tPA—We explored the possibility that the kringle-2 domain of tPA mediates the reported protein-protein interaction between tPA and PDGF-CC (11). Ni-NTA beads were therefore coated with recombinant His_6-tagged full-length PDGF-CC, and serum-free conditioned medium from COS-1 cells transfected with the different tPA truncation mutants was added. Following extensive washing, bound His_6-tagged PDGF-CC protein was specifically eluted with an imidazole-containing buffer, and the eluates were analyzed by immunoblotting using specific antibodies. The results showed that full-length PDGF-CC-coated beads specifically bound all tPA mutants except the shortest $tPA_{\Delta 300}$ mutant lacking the kringle-2 domain (Fig. 6, *two upper panels*, co-eluted tPA *above* and eluted full-length PDGF-C *below*). Similarly, experiments using Ni-NTA beads separately coated with the recombinant free His_6-tagged CUB domain (Fig. 6, *two middle panels*, co-eluted tPA *above* and CUB *below*) or the recombinant growth factor domain of PDGF-C (Fig. 6, *two lower panels*, co-eluted tPA *above* and core PDGF-C *below*), showed that both domains failed to interact with tPA when kringle-2 had been removed. Uncoated beads were used to ensure specific interaction of the tPA mutants with PDGF-CC, illustrated here by incubation of uncoated Ni-NTA beads with tPA_R. These data imply that the kringle-2 domain of tPA interacts with both the CUB and the growth factor domains of PDGF-C, thus properly positioning the tribasic -RKSR- cleavage site in the hinge region of PDGF-C and the protease domain of tPA closely together.

Regulation of PDGF-C, tPA, and PAI-1 Expression in Mouse Primary Fibroblasts—Our previous findings that PDGF-CC and tPA create a growth-stimulatory loop important for the establishment of primary fibroblast cultures might have implications in wound-healing processes, especially in the healing of chronic diabetic wounds known to have impaired granulation tissue formation probably because of reduced fibroblast activity (24). Interestingly, the expression of tPA and its inhibitor PAI-1 has been found to be altered in diabetic patients (25), but thus far, there are no reports on altered expression and activation of PDGF-CC in diabetes. To determine whether the expression of PDGF-C, and also tPA and PAI-1, is regulated in normal primary fibroblasts by metabolic conditions involved in the pathogenesis of diabetes, such as high glucose and hypoxia, we isolated primary murine fibroblasts, plated them at subconfluence, and treated them for 24 h in serum-free media. As fibroblast function is controlled by the intricate interaction of a number of growth factors, we also investigated whether the expression of PDGF-C, tPA, and PAI-1 in primary fibroblasts were regulated by such growth factors, namely TGF-β_1 and FGF-2. Following treatment the conditioned serum-free media were collected, proteins were precipitated, and the expression levels were analyzed by immunoblotting using specific antibodies and compared with a non-treated control (Fig. 7, representative blot of 5–7 individual experiments). The results showed that glucose did not affect the expression levels of PDGF-C, tPA_I, and PAI-1, whereas hypoxia decreased the expression of PDGF-C and tPA. On the other hand, stimulation of the primary fibroblasts with TGF-β_1 drastically enhanced the secretion of PDGF-C as well as PAI-1, whereas the expression of tPA was only modestly increased. In support of our findings it has previously been shown that PAI-1 transcripts are often up-regulated in hypoxic conditions and by TGF-β_1 treatment, whereas tPA transcripts are down-regulated by hypoxia and differentially regulated by TGF-β_1 in a cell-specific context (26–29). Treatment of the primary fibroblasts with FGF-2, which is known to induce expression of PDGF-C transcripts from vascular smooth muscle cells (30), did not alter the expression of PDGF-C but rather altered the expression of tPA. Taken together these results indicate that the fibroblastic PDGF-CC/tPA growth-stimulatory loop can be regulated by metabolic conditions and other growth factors, which are of importance in the pathophysiology of diabetes.

DISCUSSION

The discovery of the novel PDGFR-α ligand PDGF-CC (1) was not completely unexpected, as gene deletion studies of the classical PDGFs and the PDGFR-α had raised the possibility of an undiscovered ligand (reviewed in Ref. 31). However, the finding that PDGF-C had a unique two-domain structure and that the activity was regulated by extracellular cleavage was unpredicted (1). Until recently little was known about the protease responsible for the activation of PDGF-CC, but we have shown that the fibrinolytic serine protease tPA specifically cleaves and activates PDGF-CC (11). In this study we further investigated the molecular mechanism behind the tPA-mediated activation of PDGF-CC.

Gene deletion studies of PDGF-C have shown that PDGF-CC plays a specific role in PDGFR-α signaling and that PDGF-CC and PDGF-AA are the major PDGFR-α ligands *in vivo* (3). In our attempt to determine what is required for PDGF-CC to be a PDGFR-α agonist, we found that PDGF-CC truncation mutants with N-terminal extensions exceeding 40 amino acid residues from the growth factor domain could not activate PDGFR-α. This is consistent with the observation that mutant PDGF-AA, in which the propeptide could not be removed because of a mutation in the processing site, does not bind or activate the PDGF α-receptor (32). Within the last 40 amino acids of the hinge region in PDGF-C we have previously shown

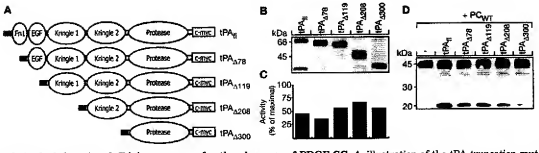

FIG. 5. **The kringle-2 domain of tPA is necessary for the cleavage of PDGF-CC.** *A*, illustration of the tPA truncation mutants used to determine the structural requirements of tPA for proteolysis of PDGF-CC. *B*, all mutants were efficiently expressed in the conditioned serum-free medium from transfected COS-1 cells as assessed by immunoblotting analysis using polyclonal anti-*c-myc* antibodies. *C*, functional analysis of the tPA truncation mutants. A tPA Spectrozyme substrate was added to buffer-exchanged conditioned media from COS-1 cells transfected with the tPA mutants, and after 3 h the formation of *p*NA was measured photometrically at 405 nm. All mutants induced the formation of *p*NA confirming preserved enzymatic activity of the truncated proteases. Purified tPA was used to define maximal *p*NA formation. *D*, COS-1 cells were co-transfected with the corresponding tPA truncation mutant in the presence of PC$_{WT}$. Serum-free conditioned media were collected 48 h after transfection, proteins were trichloroacetic acid-precipitated, and PDGF-C was detected by immunoblotting using specific polyclonal antibodies. Co-expression of PC$_{WT}$ with all tPA truncation mutants, except the shortest, generated a 22-kDa protected PDGF-C fragment.

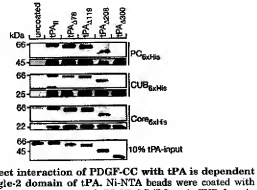

FIG. 6. **Direct interaction of PDGF-CC with tPA is dependent on the kringle-2 domain of tPA.** Ni-NTA beads were coated with recombinant His$_6$-tagged full-length PDGF-CC (*PC$_{fl\text{-}His}$*), CUB domain (*CUB$_{6xHis}$*), and growth factor domain (*Core$_{6xHis}$*) of PDGF-C expressed using the baculovirus expression system. The coated beads were incubated with conditioned serum-free medium from COS-1 cells transfected with the tPA truncation mutants illustrated in Fig. 5*A*. Part of the transfected media was analyzed by immunoblotting before its addition to the coated beads to ensure that equal relative amounts of the tPA mutant proteins were being added (10% tPA input). Following incubation with tPA the beads were thoroughly washed and the His$_6$-tagged proteins specifically eluted from the beads using a buffer containing 400 mM imidazole. Co-elution of interacting tPA species was analyzed by immunoblotting using polyclonal anti-*c-myc* antibodies. To detect elution of the PDGF-C species the blots were subsequently stripped and reblotted with PDGF-C-specific antibodies (for detection of PC$_{6xHis}$ and Core$_{6xHis}$) or alternatively anti-His antibodies (detection of CUB$_{6xHis}$).

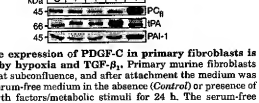

FIG. 7. **The expression of PDGF-C in primary fibroblasts is modulated by hypoxia and TGF-β_1.** Primary murine fibroblasts were seeded at subconfluence, and after attachment the medium was changed to serum-free medium in the absence (*Control*) or presence of various growth factors/metabolic stimuli for 24 h. The serum-free medium was then collected, and the proteins were trichloroacetic acid-precipitated and subjected to SDS-PAGE under reducing conditions. Immunoblot analysis using specific antibodies against PDGF-C, tPA, or PAI-1 revealed that hypoxia and TGF-β_1 modulate the PDGF-C/tPA axis.

that tPA-mediated cleavage of murine PDGF-CC occurs at, or at least around, a conserved tribasic region (amino acid residues Lys^{231}-Lys^{232}-Ser^{233}-Lys^{234}) (11). Here we confirm that this conserved tribasic region is also the site of cleavage in human PDGF-CC (amino acid residues Arg^{231}-Lys^{232}-Ser^{233}-Arg^{234}) and more specifically that the cleavage depends on Arg^{231}. It is well known that tPA cleaves plasminogen at the Arg^{561}–Val^{562} bond to produce plasmin (33), but previous findings have reported a lack of absolute specificity of tPA for an Arg-Val bond (34), and also the other non-plasminogen substrate for tPA, the NR1 subunit of the *N*-methyl-D-aspartate receptor, was recently shown to be cleaved at the Arg^{260}–Tyr^{261} bond (35). Whether tPA specifically cleaves human PDGF-CC at the Arg^{231}–Lys^{232} bond remains to be established.

A CUB domain is a common structural module found in many different kinds of proteins and is believed to participate in protein-protein or protein-carbohydrate interactions (36). The ability of the CUB domain of PDGF-C to interact with tPA and act as a competitive inhibitor of tPA-mediated proteolysis may explain the relatively low efficiency of the activation by tPA in the co-transfection assays. The stoichiometry of the activation reaction is such that generation of each molecule of receptor-active PDGF-C dimer will generate two molecules of the inhibitory free CUB domain. Whether this autoregulatory mechanism is used *in vivo* is unknown at present, but it may provide a potent regulatory mechanism controlling the activation of PDGF-CC. Apart from limited proteolysis, alternative splicing of the gene encoding PDGF-C can potentially also generate the free CUB domain of PDGF-C. However, bioinformatic efforts using the expressed sequence tag data base at NCBI have so far failed to provide any evidence of alternatively spliced PDGF-C transcripts encoding free CUB domains only.[2] The inhibitory effect of CUB on tPA activity may have clinical implications, *e.g.* in management of the bleeding side-effects often seen when using tPA in thrombolytic treatment.

The different domains of tPA have been reported to mediate interaction between tPA and various proteins, *e.g.* the finger domain binds fibrin (14) and annexin II (37, 38), and the kringle domains, in particular the second kringle domain, also bind fibrin (13, 14). Our results show that the interaction of tPA with PDGF-CC is mediated through specific interaction of the kringle-2 domain. The kringle-2 interacts with both the CUB and the growth factor domains of PDGF-CC, possibly allowing the hinge region to loop out and thus positioning the cleavage site such that the protease domain in tPA can cleave. The kringle-2 domain has been demonstrated to inhibit FGF-2-induced endothelial cell proliferation and migration (39, 40), and recently PDGF-CC has been shown to have a direct stimulatory effect on endothelial cell migration (6). As FGF-2 up-

[2] U. Eriksson, unpublished observation.

regulates PDGF-C transcription in vascular smooth muscle cells (30) it is possible that part of the inhibitory effect of the kringle-2 domain is through binding and subsequent blockage of PDGF-CC activation. Furthermore, we could speculate that upon the interaction of PDGF-CC with kringle-2, the other domains of tPA could interact with molecules such as the low density lipoprotein receptor-related protein, known to both interact with tPA (41) and control PDGFR signaling (42) and thereby facilitating a localized generation of active PDGF-CC. Interestingly, tPA induces blood-brain barrier opening via interaction with the low density lipoprotein receptor-related protein and proteolysis of an as yet unidentified substrate (43).

Despite the similarities between PDGF-CC and the novel PDGFR-β ligand, PDGF-DD, tPA fails to interact and induce cleavage of this latter factor (11). Thus far, less is known about the activation of PDGF-DD. We have suggested previously that the genes for the classical and novel PDGFs separated early during evolution and that the novel PDGFs then arose from a common ancestor (44). It is therefore not unlikely that the protease involved in PDGF-DD activation has a similar structural organization as tPA. Using a computer-based strategy we could identify several serine proteases with similar domain organization as tPA, including the other plasminogen activator, uPA (urokinase PA). Whether any of these homologous proteases can cleave and activate PDGF-DD remains to be established.

Non-healing foot ulcers in diabetic patients are a common and expensive complication partially caused by reduced fibroblast activity (24). To develop rational therapeutic strategies it has become a major priority to characterize the pathophysiological mechanism of the delayed wound healing and the impaired fibroblast activity in diabetic patients. Considering that the growth of primary fibroblasts in culture partially depends on a PDGF-CC/tPA stimulatory loop, we hypothesized that dysregulation of PDGF-CC signaling might be involved in the impaired function of fibroblasts seen in diabetic wounds. Our experiments demonstrate that hyperglycemia *per se* does not alter expression of the PDGF-C/tPA axis in normal primary fibroblasts but that hypoxia, known to play an important role in all diabetes complications (45), decreases the expression of PDGF-C and thus a mitogenic signal for fibroblasts. It is worth noting that hyperbaric oxygen therapy is used to accelerate the rate of healing of diabetic foot ulcers (46). Furthermore, we show that treatment with TGF-β_1 greatly induced the expression of PDGF-C in the primary fibroblasts, which is interesting considering that transgenic overexpression of either PDGF-C (4) or TGF-β_1 (47) in mouse heart results in fibrosis and cardiac hypertrophy. Although it is known that ectopically applied PDGF-CC can enhance delayed wound healing in diabetic mice (21) it still remains to be established whether PDGF-C expression is impaired in diabetic conditions. In summary, the described molecular mechanism by which PDGF-CC becomes a PDGFR-α agonist through tPA-mediated proteolysis will assist the understanding of PDGF signaling in normal and pathological conditions.

Acknowledgment—We thank Aristidis Moustakas for the kind gift of TGF-β_1.

REFERENCES

1. Li, X., Pontén, A., Aase, K., Karlsson, L., Abramsson, A., Uutela, M., Bäckström, G., Hellström, M., Boström, H., Li, H., Soriano, P., Betsholtz, C., Heldin, C. H., Alitalo, K., Ostman, A., and Eriksson, U. (2000) *Nat. Cell Biol.* **2**, 302–309
2. Heldin, C. H., and Westermark, B. (1999) *Physiol. Rev.* **79**, 1283–1316
3. Ding, H., Wu, X., Boström, H., Kim, I., Wong, N., Tsoi, B., O'Rourke, M., Koh, G. Y., Soriano, P., Betsholtz, C., Hart, T. C., Marazita, M. L., Field, L. L., Tam, P. P., and Nagy, A. (2004) *Nat. Genet.* **36**, 1111–1116
4. Pontén, A., Li, X., Thorén, P., Aase, K., Sjöblom, T., Ostman, A., and Eriksson, U. (2003) *Am. J. Pathol.* **163**, 673–682
5. Eitner, F., Ostendorf, T., Kretzler, M., Cohen, C. D., Eriksson, U., Grône, H. J., and Floege, J. (2003) *J. Am. Soc. Nephrol.* **14**, 1145–1153
6. Li, X., Tjwa, M., Moons, L., Fons, P., Noel, A., Ny, A., Zhou, J. M., Lennartsson, J., Li, H., Luttun, A., Pontén, A., Devy, L., Bouché, A., Oh, H., Manderveld, A., Blacher, S., Communi, D., Savi, P., Bono, F., Dewerchin, M., Foidart, J. M., Autiero, M., Herbert, J. M., Collen, D., Heldin, C. H., Eriksson, U., and Carmeliet, P. (2005) *J. Clin. Investig.* **115**, 118–127
7. Cao, R., Bråkenhielm, E., Li, X., Pietras, K., Widenfalk, J., Ostman, A., Eriksson, U., and Cao, Y. (2002) *FASEB J.* **16**, 1575–1583
8. Bergsten, E., Uutela, M., Li, X., Pietras, K., Ostman, A., Heldin, C. H., Alitalo, K., and Eriksson, U. (2001) *Nat. Cell Biol.* **3**, 512–516
9. LaRochelle, W. J., Jeffers, M., McDonald, W. F., Chillakuru, R. A., Giese, N. A., Lokker, N. A., Sullivan, C., Boldog, F. L., Yang, K., Vernet, C., Burgess, C. E., Fernandes, E., Deegler, L. L., Rittman, B., Shimkets, J., Shimkets, R. A., Rothberg, J. M., and Lichenstein, H. S. (2001) *Nat. Cell Biol.* **3**, 517–521
10. Li, X., and Eriksson, U. (2003) *Cytokine Growth Factor Rev.* **14**, 91–98
11. Fredriksson, L., Li, H., Fieber, C., Li, X., and Eriksson, U. (2004) *EMBO J.* **23**, 3793–3802
12. Collen, D. (2001) *Hematology (Am. Soc. Hematol. Educ. Program)*, 1–9
13. Verheijen, J. H., Caspers, M. P., Chang, G. T., de Munk, G. A., Pouwels, P. H., and Enger-Valk, B. E. (1986) *EMBO J.* **5**, 3625–3630
14. van Zonneveld, A. J., Veerman, H., and Pannekoek, H. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 4670–4674
15. rtPA Stroke Study Group. (1995) *N. Engl. J. Med.* **333**, 1581–1587
16. Qian, Z., Gilbert, M. E., Colicos, M. A., Kandel, E. R., and Kuhl, D. (1993) *Nature* **361**, 453–457
17. Carroll, P. M., Tsirka, S. E., Richards, W. G., Frohman, M. A., and Strickland, S. (1994) *Development (Camb.)* **120**, 3173–3183
18. Vepes, M., Sandkvist, M., Coleman, T. A., Moore, E., Wu, J. Y., Mitola, D., Bugge, T. H., and Lawrence, D. A. (2002) *J. Clin. Investig.* **109**, 1571–1578
19. Yepes, M., and Lawrence, D. A. (2004) *Trends Cardiovasc. Med.* **14**, 172–180
20. Nicole, O., Docagne, F., Ali, C., Margaill, I., Carmeliet, P., MacKenzie, E. T., Vivien, D., and Buisson, A. (2001) *Nat. Med.* **7**, 59–64
21. Gilbertson, D. G., Duff, M. E., West, J. W., Kelly, J. D., Sheppard, P. O., Hofstrand, P. D., Geo, Z., Shoemaker, A., Kaufman, S. A., Moore, M., Feldhaus, A. L., Humes, J. M., Palmer, T. E., and Hart, C. E. (2001) *J. Biol. Chem.* **276**, 27406–27414
22. Green, S., Issemann, I., and Sheer, E. (1988) *Nucleic Acids Res.* **16**, 369
23. Eriksson, A., Siegbahn, A., Westermark, B., Heldin, C. H., and Claesson-Welsh, L. (1992) *EMBO J.* **11**, 543–550
24. Hehenberger, K., Kratz, G., Hansson, A., and Brismar, K. (1998) *J. Dermatol. Sci.* **16**, 144–151
25. Eliasson, M. C., Jansson, J. H., Lindahl, B., and Stegmayr, B. (2003) *Cardiovasc. Diabetol.* **2**, 19
26. Pinsky, D. J., Liao, H., Lawson, C. A., Yan, S. F., Chen, J., Carmeliet, P., Loskutoff, D. J., and Stern, D. M. (1998) *J. Clin. Investig.* **102**, 919–928
27. Schacke, W., Beck, K. F., Pfeilschifter, J., Koch, P., and Hattenbach, L. O. (2002) *Investig. Ophthalmol. Vis. Sci.* **43**, 2799–2805
28. Docagne, F., Nicole, O., Marti, H. H., MacKenzie, E. T., Buisson, A., and Vivien, D. (1999) *FASEB J.* **13**, 1315–1324
29. Saed, G. M., and Diamond, M. P. (2003) *Fertil. Steril.* **79**, 164–168
30. Midgley, V. C., and Khachigian, L. M. (2004) *J. Biol. Chem.* **279**, 40289–40295
31. Kazlauskas, A. (2000) *Nat. Cell Biol.* **2**, E78–E79
32. Siegfried, G., Khatib, A. M., Benjannet, S., Chrétien, M., and Seidah, N. G. (2003) *Cancer Res.* **63**, 1458–1463
33. Castellino, F. J., and Powell, J. R. (1981) *Methods Enzymol.* **80**, 365–378
34. Ding, L., Coombs, G. S., Strandberg, L., Navre, M., Corey, D. R., and Madison, E. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7627–7631
35. Fernández-Monreal, M., López-Atalaya, J. P., Benchenane, K., Cacquevel, M., Dulin, F., Le Caer, J. P., Rossier, J., Jarrige, A. C., MacKenzie, E. T., Colbeck, N., Ali, C., and Vivien, D. (2004) *J. Biol. Chem.* **279**, 50850–50856
36. Bork, P., and Beckmann, G. (1993) *J. Mol. Biol.* **231**, 539–545
37. Beebe, D. P., Miles, L. A., and Plow, E. F. (1989) *Blood* **74**, 2034–2037
38. Hajjar, K. A., Jacovina, A. T., and Chacko, J. (1994) *J. Biol. Chem.* **269**, 21191–21197
39. Kim, H. K., Lee, S. Y., Oh, H. K., Kang, B. H., Ko, H. J., Lee, Y., Shin, J. Y., Hong, Y. K., and Joe, Y. A. (2003) *Biochem. Biophys. Res. Commun.* **304**, 740–746
40. Carroll, V. A., Nikitenko, L. L., Bicknell, R., and Harris, A. L. (2005) *Arterioscler. Thromb. Vasc. Biol.* **25**, 736–741
41. Bu, G., Williams, S., Strickland, D. K., and Schwartz, A. L. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 7427–7431
42. Boucher, P., Gotthardt, M., Li, W. P., Anderson, R. G., and Herz, J. (2003) *Science* **300**, 329–332
43. Yepes, M., Sandkvist, M., Moore, E. G., Bugge, T. H., Strickland, D. K., and Lawrence, D. A. (2003) *J. Clin. Investig.* **112**, 1533–1540
44. Fredriksson, L., Li, H., and Eriksson, U. (2004) *Cytokine Growth Factor Rev.* **15**, 197–204
45. Cameron, N. E., Eaton, S. E., Cotter, M. A., and Tesfaye, S. (2001) *Diabetologia* **44**, 1973–1988
46. Kalani, M., Jörneskog, G., Naderi, N., Lind, F., and Brismar, K. (2002) *J. Diabetes Complicat.* **16**, 153–158
47. Seeland, U., Haeussler, G., Hinrichs, R., Rosenkranz, S., Pfitzner, T., Scharfetter-Kochanek, K., and Böhm, M. (2002) *Eur. J. Clin. Investig.* **32**, 295–303